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CHRONIC FOCAL INTERSTITIAL HEPATITIS IN THE PIG: ITS EXPERIMENTAL PRODUCTION BY FEEDING *ASCARIS* EGGS

By

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INTRODUCTION

Chronic focal interstitial hepatitis (" white spot liver " or " milk spot ") in the pig has been fully described by one of us (White, 1941) and the cause was then believed to be the passage through the liver of migrating *Ascaris* larvae. This view was supported by the finding of a second stage *Ascaris* larva in a typical lesion, and of degenerated parasites in a number of lesions, and by the obviously parasitic host reaction, which was characterised by a very intense eosinophile infiltration. Preliminary experiments to reproduce the condition in young pigs by feeding embryonated *Ascaris* eggs have been briefly reported (Oldham and White, 1942) and they supported the view of the aetiology expressed from studying the naturally occurring condition. This paper describes the results of the preliminary experiments in more detail and of a subsequent experiment on similar lines.

MATERIALS AND METHODS

For the 1940 and 1941 experiments the methods of obtaining infective material were essentially similar. Eggs were obtained directly from the uteri of dissected *Ascaris lumbricoides* females in much the same way as described by Roberts (1934). They were expelled from short lengths of uterine branches into physiological saline in which they were allowed to remain for about three days. During this period the saline was agitated a few times each day and this helped to rid the eggs of some of the albuminous coating surrounding the shell; there was thus less tendency for the eggs to stick to each other, to the petri dish containers, and to the syringe by which they were later fed to the pigs. The saline was afterwards replaced by 2 per cent. formalin, added in sufficient quantity to cover the eggs, and development proceeded in a fairly uniform manner in this fluid, which inhibited bacterial activity.

It is generally stated that, at the optimum temperature of 30° to 33° C., the first stage larva appears in the egg about the eighth day and the first moult occurs in the egg about the 18th day, after which, and not before, the egg becomes infective. It has been noted, however, that such eggs may produce but light infestations and that they must pass through a post-embryonation lag or maturation period before they become fully infective. Thus, Roberts (1934) found that " although mice were fed embryonated eggs 18 to 20 days old and macroscopic evidence of hatching and migration could be

observed after a three-day infestation period in the small intestine, in the liver, and, in the case of the older eggs, in the lungs, larvae were not isolated in any numbers from these tissues until the incubation period of the eggs had been increased to 22 days." Similarly, Stoll (1933) found that eggs from faeces or removed from the uteri of females and incubated at room temperature did not produce pulmonary symptoms in guinea-pigs till 40 days old. To ensure that the material to be fed experimentally to the pigs contained, therefore, a high proportion of infective eggs the formalin suspensions were incubated at 30° C. for about twelve weeks ; inspection, two or three times a week, showed that a relatively high proportion of the eggs, in fact, became embryonated.

Before embarking on the experiments with pigs, the eggs were tested for infectivity and known dosages of infective eggs, prepared by a dilution technique, were invariably used. Infectivity tests were carried out on rabbits and guinea-pigs since Graham (1937) had found these animals more suitable for demonstrating damage by *Ascaris lumbricoides* larvae than rats and mice. The test animals were killed three and seven days after receiving the infective eggs (suspended in a small quantity of saline) orally by a pipette and their livers and lungs respectively examined for lesions. Then the livers and lungs, removed from the freshly killed animals, were finely minced and *Ascaris* larvae were isolated from them by the Baermann apparatus. The numbers of larvae recovered in this way and the macroscopic lesions observed were taken as sufficient indication that the material to be fed to the pigs contained a large proportion of infective eggs. A somewhat larger proportion of eggs were infective in the material used in the second than in the first experiment.

The first (1940) experiment was carried out on nine pigs, eight to eleven weeks old, from two litters after weaning. They included a " ratling " (No. 2241) weighing only 11 lb., which was used to ascertain approximately the dose of embryonated eggs suitable ; it was fed 200,000 eggs. Pigs No. 2268, 2269, 2270 and 2273 received 1,600,000 eggs thoroughly mixed with a feed ; it was assumed that each pig would eat approximately equal quantities of food and eggs. The remaining animals were each separately fed 400,000 eggs by delivering them, suspended in a small quantity of saline, orally over the back of the tongue from the nozzle of a hypodermic syringe. The pigs were killed at different predetermined intervals after experimental feeding and a detailed autopsy on each was carried out, which included histological examination of pieces of liver, lung, and kidney. Several pieces (from six to 14) of liver were taken for histological examination and, at intervals of about 100μ, sections were cut in duplicate through the paraffin blocks. The first section of each pair was stained with haematoxylin and eosin and the second kept in reserve so that it could later be stained if evidence of a parasite was found in the first section. The same procedure was adopted when lesions were present in the lung and kidney. Just

before the pigs were slaughtered an ear vein was pricked for blood and in the film prepared the number of eosinophils in 1,000 leucocytes was counted.

Immediately after *post-mortem* examination the finely minced liver and lungs were placed in a Baermann apparatus to extract any larvae present. With some carcases, the peritoneal and thoracic cavities and the trachea were washed out with saline and the sediments from the washings, after decanting the supernatant fluid, were examined for larvae under a binocular dissecting microscope, as were also the extractions from the Baermann apparatus. In addition, when each pig was killed, a small amount of faeces from the rectum was examined for helminth eggs by a sugar-flotation-centrifugation technique.

The second (1941) experiment was carried out on eleven weaned pigs, of which nine were gilts. They comprised the litter of one sow and were eleven weeks old when received. Since the preceding experiment had been complicated by the presence of immature ascarids in the intestine of some of the pigs, for this second experiment an attempt was made to obtain worm-free animals. The faeces of several sows which had litters were examined microscopically on three occasions at weekly intervals and the litter selected came from a sow whose faeces had been negative for helminth eggs at each examination.

The procedure was similar to that of the previous experiment except that blood films were made when the animals were fed with *Ascaris* eggs as well as at slaughter. The Baermann method of extracting larvae from the tissues was also modified. In the first experiment the minced liver and lung tissue was supported on wire sieves and butter muslin placed in 6-inch diameter glass funnels and larval extraction allowed to proceed at laboratory temperature. The water, used for extraction, was raised to 40° to 45° C. but probably cooled rapidly and consequently the larvae soon lost their initial activity so that comparatively few were isolated. In the second experiment, larger, 8·5-inch metal funnels were used so that the minced lung and liver tissue was spread more thinly over the muslin covered screen. The extraction was also continued longer, for at least four hours, in some cases overnight, in a room thermostatically controlled at 38° C. On the whole, more larvae were isolated from the tissues in the second experiment but the numbers were small in comparison with the large numbers of eggs fed to the pigs. The efficiency of the Baermann apparatus, according to Dinaburg (1942), varies with the type of material used as substrate and the initial number of larvae. It appears that most investigations using this technique have been carried out with soils of differing consistency or with faeces; in Dinaburg's investigations, whether relatively few or large numbers of larvae were involved, the smallest percentage of larvae recovered was from faeces. That we did not recover large numbers of *Ascaris* larvae from the Baermann extractions is

probably due, at least in part, to the consistency of the minced liver and lung tissue which differed from that of faeces and much impeded freedom of larval movement. Thus, larvae would escape from the peripheral material into the fluid whereas those in the innermost layers would remain imprisoned. We were, however, not concerned so much with recovering large numbers of larvae as with finding out whether or not larvae were present in various tissues and with observing the course of their migrations, and particularly their occurrence in the liver.

EXPERIMENT 1

Protocols

Pig 2241 (" ratling ").—Killed 48 hours after receiving 200,000 eggs by pipette. Blood showed 1·3 per cent. eosinophils at the time of dosing and 4·7 per cent. when killed. Liver showed about two dozen small greyish foci, none larger than a pin head, on each surface, some with a haemorrhagic centre, and similar lesions in the parenchyma. No other macroscopic abnormality. The small intestine contained 15 immature *Ascaris*, nine female and six male. No larvae were isolated from the liver, lungs (subsequent experience showed that this did not mean their absence in the tissues) or the peritoneal and thoracic cavity washings.

The liver on section showed interlobular septa which were poorly developed for a pig eleven weeks old: there were numerous small focal aggregations of eosinophils and neutrophils and in one field there was an oblique section through a parasite, about 10 × 30μ, and surrounded by a collection of eosinophils (Fig. 5). Within the parasite were small blue dots similar to those seen in sections of the parasites encountered in natural cases of " milk spot " (White, 1941). Nothing abnormal was noted in sections of lung or kidney. The peripheral sinuses of the mesenteric lymph nodes were packed with eosinophils but no parasites were seen in them.

Pig 2236.—Killed six days after receiving 400,000 eggs. Blood films at slaughter showed 3·4 per cent. eosinophils. The liver on both surfaces showed about 30 whitish foci, some with a haemorrhagic centre and similar lesions, somewhat less easily seen, throughout its substance. The periphery of most lesions consisted of greyish-white, thickened, interlobular septa as in natural cases of " milk spot." The lungs showed a few areas of suppurative bronchopneumonia. The small intestine contained a single immature male *Ascaris*. Extraction of the lungs yielded *Ascaris* larvae: no larvae were obtained from the liver or from tracheal washings.

The liver lesions showed on section a heavy eosinophile infiltration, fibroblast proliferation in the interlobular septa, and typical superficial and deep " milk spots." In some lesions there were small lymphoid foci, two of which showed a cross section of a parasite surrounded by a pink staining fibrillary material. Those lesions, which on macroscopical examination showed a haemorrhagic centre, were seen to consist of a necrotic and haemorrhagic central area which involved two or three lobules and was heavily infiltrated with eosinophils (Fig. 9). The surrounding septa showed fibroblast proliferation. The consolidated areas of lung were affected with catarrhal and suppurative bronchopneumonia and bronchitis. In other parts of the lung there were focal collections of eosinophils and a few multinucleated giant cells within the alveolar spaces. In two such areas there were cross sections of parasites, about 30 × 40μ.

Pig 2235.—Kept as a control and killed at the same time as the preceding. Blood films prepared at the time of killing showed 1·6 per cent. eosinophils. The liver was normal except for a few small areas in which the interlobular septa were slightly thickened, and on section showed some fibrosis and eosinophile infiltration, probably due to spontaneous infestation with small numbers of *Ascaris* larvae during the suckling period. The other organs were normal. No *Ascaris* were found in the intestines. The findings of other workers suggest that this by no means excludes the possibility of migration of *Ascaris* larvae, only a small proportion of which would reach the adult stage in the intestine.

Four pigs, Nos. 2269, 2270, 2273 and 2268, were fed 1,600,000 eggs in all in a gallon of food, after having been fasted for the preceding 16 hours. All four animals consumed the food readily but the exact proportion of *Ascaris* eggs ingested by each pig was not known. The pigs were killed at intervals.

Pig 2269.—Killed three days after feeding. Blood films at slaughter showed 6·9 per cent. eosinophils. The liver showed about 100 greyish foci on each surface, many having a haemorrhagic centre. No larvae were seen in three lesions examined after crushing on a slide and none was found by Baermann extraction. One *Ascaris* larva was obtained from the lungs by extraction; none was recovered from the peritoneal cavity washings. There were no adult *Ascaris* in the intestines.

The liver lesions were typical early " milk spots," with central parenchyma destruction. In one section a complete *Ascaris* larva was seen, cut longitudinally and measuring 10 × 200μ. (Fig. 4.) Its path of migration was suggested by a track of degenerating liver cells and leucocytes. The interlobular septa were infiltrated with some eosinophils, which were present also in enormous numbers in the central haemorrhagic zone of the lesions. Sections of the other organs revealed nothing abnormal.

Pig 2270.—Killed six days after feeding. Blood films at slaughter showed 7·1 per cent. eosinophils. The liver showed on each surface about 30 lesions which were larger than in Pig 2269 and for the most part had no haemorrhagic centre. In the lungs there were numerous petechial haemorrhages and some oedema. The other organs were normal. There were two immature female *Ascaris* in the intestine. Tracheal washings and liver extraction yielded no larvae but some were found by lung extraction.

The liver lesions were seen on section to contain greatly thickened interlobular septa with much fibroblast proliferation and heavily infiltrated with eosinophils (Fig. 11). In one lesion the septa reached 500μ in width, compared with the normal of about 10μ. In some foci there were collections of lymphoid cells within the thickened septa. The lungs showed peribronchial and alveolar haemorrhage and free erythrocytes in the alveolar spaces, as well as a general increase in eosinophils, which were numerous within the lumen of the small blood vessels some of which contained thrombi.

Pig 2271.—This was a control pig, killed at the same time as the preceding. The eosinophile count was 2·2 per cent. There were no macroscopic lesions in any of the organs and no *Ascaris* in the intestine. Sections showed no abnormality.

Pig 2273.—Killed 13 days after feeding. Blood films showed 15·7 per cent. eosinophils. In the liver there were several, scarcely visible, diffuse " milk spots " which had no opaque centre, as well as five superficial opaque lesions similar in appearance to the " pearly spots " seen in natural cases of

" milk spot liver." Liver and lung extractions and tracheal washings were negative for larvae.

The circumscribed foci in the liver were seen on section to consist of aggregations composed of numerous " follicles " of lymphoid cells with few eosinophils which were confined to the periphery. The surrounding interlobular septa were only slightly thicker than normal. The lungs showed massive peribronchial collections of eosinophils.

Pig 2272.—Control pig, killed at the same time as the preceding. Blood films showed 2·4 per cent. eosinophils. The liver and other organs were normal, both macroscopically and histologically. The small intestine contained four immature *Ascaris*.

Pig 2268.—Killed 27 days after feeding. The peritoneal cavity contained an excessive amount of clear fluid and there were firm fibrous adhesions between the parietal and visceral layers of peritoneum. The liver showed about 30 superficial lesions, some being of the " pearly " circumscribed type and 1 to 2 mm. in diameter, whilst the others were rather indistinct, diffuse " milk spots." There were also a few lesions of both types in the substance of the liver. The other organs were normal. Blood films showed 2·0 per cent. eosinophils. The small intestine yielded four immature *Ascaris*, three female and one male. The tracheal washings and lung extraction were negative for larvae but larvae were demonstrated in the intestinal contents.

The circumscribed liver lesions were found on section to consist of lymphoid nodules with numerous " follicles " (Fig. 8); a section through the middle of the largest lesion included 25 individual " follicles." The cause of the peritoneal adhesions was not determined. The diffuse indistinct lesions consisted of thickened interlobular septa, infiltrated with eosinophils, and probably represented old " milk spots." In the lungs there were numerous peribronchial collections of eosinophils.

Discussion of Results

This preliminary experiment showed that lesions identical in appearance with those occurring in natural " white spot liver " could be produced in young pigs by feeding embryonated *Ascaris* eggs. In the early stages the lesions showed a central haemorrhagic zone with liver cell destruction and intense eosinophile infiltration, followed by absorption and repair and fibroblast proliferation in the neighbouring interlobular septa. Old lesions showed persistent thickening of the septa and eosinophile infiltration. The formation of lymphoid tissue in the lesions became evident, the larger masses of such tissue comprising collections of spherical " follicles " such as have been described in the natural condition.

Although at first sight most of the lesions appeared to be on the surface of the liver, many deep lesions could be demonstrated by cutting thin sections of the organ. The lesions on the anterior and posterior surfaces were roughly similar in number; this did not lend support to the view that invasion is by the peritoneal route but rather by the haematogenous route, namely, along the portal vein, which view upon the mode of travel of *Ascaris* from the small intestine is now generally accepted. An attempt to demonstrate larvae in the peritoneal washings failed.

The results were complicated by the finding of immature *Ascaris* in the small intestine of some animals; this indicated infestation before arrival at the Institute. The changes discoverable after feeding *Ascaris* eggs, taken in conjunction with the findings in the control pigs, left no doubt that the numerous lesions could be reasonably attributed to the heavy artificial infestation. It was decided, however, to repeat the experiment using, if they could be secured, weaned pigs free from *Ascaris* infestation.

EXPERIMENT 2

Protocols

Two pigs, 2736 and 2738, were selected at random and weighed, blood films were made, and each animal was given 250,000 eggs by the mouth by means of a syringe.

Pig 2736.—At the time of feeding, the eosinophils were 1·7 per cent.; when the pig was killed 24 hours later the eosinophile count was 4·9 per cent. The liver showed numerous petechial haemorrhages on both surfaces, more numerous on the anterior than on the posterior surface. Similar haemorrhages were seen in the depth of the liver and were more prominent after fixation in formalin. The lungs were normal. Numerous petechiae were seen in the mucosa of the small intestine. Peritoneal washings and blood from the portal vein both failed to yield any *Ascaris* larvae, and none was found in extractions of the liver and lungs.

The petechiae in the liver were found on section to be mainly situated in the intermediate zone of the lobules. Only one of the lesions examined showed eosinophile infiltration, the others showing degenerating liver cells, free erythrocytes, and a few neutrophil leucocytes. There was also a single old diffuse "milk spot," previously recognised on macroscopic examination; this showed thickened septa, with fibrosis, eosinophile infiltration and aggregations of lymphoid cells. The other organs were normal.

Pig 2738.—At the time of feeding the eosinophile count was 1·4 per cent. and it had risen to 4·5 per cent. when the pig was killed six days later. Examination of the faeces four days after the eggs had been fed showed embryonated *Ascaris* eggs containing dead larvae and also some non-embryonated infertile eggs. The liver at autopsy showed 24 lesions on the posterior surface and 44 on the anterior surface, as well as many lesions in the depth of the organ. The main change in all lesions was thickening of the septa, which were greyish yellow in contrast to the whitish colour of older lesions. Two lesions were crushed and examined in the fresh state and one revealed an *Ascaris* larva at the same stage of development as that isolated from a natural case of "milk spot liver" and illustrated in an earlier paper (White, 1941). There were small areas of lobular consolidation in the lungs. There were two small whitish foci in the cortex of the right kidney. Two immature adult *Ascaris* were found in the small intestine. Extraction of the lungs yielded many *Ascaris* larvae: none was found in the liver. The only specimens found in faeces taken from the rectum at autopsy were forage mites and mite eggs.

The liver lesions consisted of foci of central parenchyma destruction, surrounded by thickened septa heavily infiltrated with eosinophils. Early

fibroblast proliferation was already evident and in one lesion there were small collections of lymphoid cells. The lungs showed areas of non-parasitic bronchopneumonia.

Pig 2742.—Killed 18½ hours after receiving 250,000 eggs. At the time of feeding the eosinophile count was 1·9 per cent.; blood taken just before slaughter failed to show a single eosinophile in 2,000 leucocytes. The liver showed one old superficial lesion and no signs of recent damage. There were three small areas of lobular consolidation in the lungs. There were no petechiae in the intestine, the contents of which contained two adult *Ascaris*, one male and one female. The following were examined for *Ascaris* larvae: peritoneal washings, blood from the portal vein and posterior vena cava, extractions of liver, lungs, and mesenteric lymph nodes. No larvae were found. Faeces taken from the rectum at autopsy showed *Ascaris* eggs of two kinds, some derived probably from the mature *Ascaris* found in the intestine and others from the experimental dose. The former were distinguished by their albuminous covering.

There were no histological changes in the liver and the lung lesions consisted of a non-parasitic bronchopneumonia. The mesenteric lymph nodes showed large numbers of eosinophils and neutrophils, especially numerous in the peripheral sinuses.

Pig 2733.—Killed four days after receiving 250,000 eggs. The eosinophile count was 2·6 per cent. at the time of feeding and 10·6 per cent. just before slaughter. The liver showed about 100 lesions on each surface (Fig. 1) and very many in the depth of the organ. A haemorrhagic centre was present in some, but not all, of the lesions. An *Ascaris* larva was found in a fresh preparation made by crushing one of the lesions. The lungs were consolidated in their antero-inferior portions but no haemorrhages were seen. A fresh preparation of the lung showed a moulted larval skin but no larvae were demonstrated in any of several preparations. The other organs were normal. There were no *Ascaris* in the intestine and no *Ascaris* eggs were found in the faeces. Extractions of the liver, lungs, and mesenteric nodes, peritoneal washings, and blood from the portal vein and posterior vena cava all failed to yield any larvae.

Sections of the liver lesions showed parenchyma destruction, intense eosinophile infiltration, some neutrophils, and signs of early fibroblast proliferation. Superficial lesions were covered by a thickened Glisson's capsule, heavily infiltrated with eosinophils. Several small branches of the portal vein were infiltrated with eosinophils and showed an acute phlebitis. The consolidated areas of lung showed a non-parasitic bronchopneumonia.

Pig 2739 S.—Killed ten days after receiving 250,000 eggs. Eosinophils in the blood at the time of feeding were 3·0 per cent.; just before slaughter the level was the highest recorded, 23·7 per cent. The liver showed about 100 greyish foci on each surface (Fig. 2), and a very large number throughout the depth of the organ. There were also several lesions with a haemorrhagic centre which appeared to be of more recent origin. There were many small haemorrhages in the lungs as well as lobular areas of blood aspiration, the latter being terminal and resulting from destruction of the animal by shooting. There were two small whitish foci in the cortex of the left kidney and one similar lesion in the right kidney. Cutting the kidneys into thin slices revealed about a dozen more lesions in each kidney. The other organs were normal. There were no adult *Ascaris* in the intestine and the rectal contents showed only forage mites and mite eggs. Tracheal washings and liver

extraction yielded no larvae ; numerous larvae were found on lung extraction and in the intestinal contents.

The liver lesions were found on section to be of various kinds, varying from recent foci with a haemorrhagic centre to older lesions with marked fibrosis. The latter contained foci of lymphoid tissue, often surrounded by a zone of eosinophils and in one such lesion a parasite was seen in section (Fig. 7). In the lungs there was very marked peribronchial and perivascular eosinophile infiltration and large numbers of eosinophils were visible within the lumen of blood vessels (Fig. 12). These changes were in patches, other areas of lung appearing normal. Erythrocytes and a few eosinophils were seen within groups of alveoli in relation to affected bronchi. One section included a cross section of a parasite. The kidney lesions consisted of focal collections of eosinophils, together with some fibroblasts and mononuclear cells. No parasites were seen in these lesions.

Pig 2739 L.—Killed 20 days after receiving 250,000 eggs. Blood films made at the time of dosing showed 1·4 per cent. eosinophils and just before slaughter the count had risen to 12·6 per cent. About 100 lesions were seen on each of the liver surfaces. They could be classified as belonging to three main types : (1) Very numerous, greyish, diffuse lesions, some having a " pearly " centre ; (2) a smaller number of cream coloured or yellowish lesions, with or without central opaque nodules, considered to be of more recent origin ; (3) occasional " pearly " nodules without obvious thickening of adjacent interlobular septa. Examples of all three types were seen in the depth of the liver. The antero-inferior parts of both lungs were consolidated. The other organs were normal. The tracheal washings and liver and lung extractions yielded no larvae. A number of larvae were found in the contents of the intestines. The faeces showed mite eggs but no *Ascaris* eggs.

Sections of the liver lesions included examples of the three types described. The first consisted of diffuse septal thickening, infiltrated with large numbers of eosinophils (Fig. 10) and sometimes with central parenchyma destruction. The second type of lesion was more recent, with massive eosinophile collections, both in the septa and within and around blood vessels. The third type consisted of lymphoid nodules, one of which showed a parasite cut twice in cross section (Fig. 6). In the lungs there was very massive infiltration of bronchi and bronchioles, as well as blood vessels, with eosinophils. There were also small intra-alveolar haemorrhages and many alveoli contained eosinophils, erythrocytes and histiocytes. The large consolidated areas showed a non-parasitic bronchopneumonia.

Pig 2734.—Killed 30 days after receiving 250,000 eggs. The eosinophile count at the time of dosing was 3·4 per cent., and at slaughter 7·2 per cent. About 100 liver lesions were present on each surface (Fig. 3) and were similar to those seen in the previous pig. There was consolidation of the antero-inferior portions of both lungs. There was a small greyish focus in the cortex of the right kidney. The small intestine contained one immature adult female *Ascaris*. Tracheal washings yielded no larvae and none was found on extraction of the lungs and liver. Extraction of the contents of the small intestine and the stomach contents yielded larvae. The faeces contained forage mites and mite eggs.

The histological features of the liver lesions were similar to those of the previous pig, except that there was more fibrosis. Some of the lymphoid nodules contained well developed " follicles " and in the centre of one such

lesion there was a pink staining structure which might have been a degenerated parasite. The lungs showed peribronchial and intra-alveolar eosinophile infiltration, as well as hyperplasia of peribronchial lymphoid tissue. The pneumonic areas were not of parasitic origin. The small lesion in the kidney consisted of lymphoid cells and could not be identified as parasitic.

Pig 2741.—Control normal pig, killed at the same time as the preceding. The eosinophils in the blood at the time of slaughter were 4·0 per cent., the highest in any animal not artificially infected. The posterior surface of the liver showed two yellowish lesions near the bile duct, considered to be of recent origin. The other organs were normal. The rectal contents contained mite eggs only. There were no intestinal *Ascaris*. Extractions of the liver, lungs and contents of the small intestine yielded no larvae.

Sections of the two liver lesions showed them to be typical recent " milk spots," with intense eosinophile infiltration. They were presumably due to accidental infection. The other portions of liver examined histologically showed nothing abnormal.

Pig 2735.—Given ten weekly doses of 25,000 eggs each and killed four days after the last dose. The eosinophile counts made at intervals are shown in Fig. 13. The liver showed numerous rather indistinct diffuse greyish lesions, a few having an opaque centre. There were also a number of fairly circumscribed whitish foci, considered to be of lymphoid nature. The depth of the liver showed similar lesions and also yellowish foci, probably of more recent origin. The cardiac lobe of the right lung was consolidated and the overlying pleura was inflamed. The cortex of the right kidney contained a small greyish focus. There was one immature adult female *Ascaris* in the small intestine. The rectal contents showed numerous fodder mites and mite eggs and some strongyle eggs ; there were no *Ascaris* eggs. Extractions of the lungs and contents of the small intestine each yielded larvae, but none was recovered from the liver.

Sections of the liver showed recent lesions with parenchyma destruction, as well as older and more diffuse foci and lymphoid nodules. In the lungs there was intense eosinophile infiltration of the bronchial walls in some areas and hyperplasia of the peribronchial lymphoid tissue. The area of non-parasitic consolidation showed the usual features. Groups of alveoli in some sections showed free eosinophils and histiocytes ; such alveoli were mainly closely related to bronchi which also showed eosinophile infiltration. The kidney lesion consisted of lymphoid cells with a follicular arrangement, together with a few eosinophils at the periphery.

Pig 2740.—Given ten weekly doses of 25,000 eggs each, followed by a single dose of 250,000 eggs, and killed six days later. The eosinophile counts are shown in Fig. 13. The liver showed about a dozen recent " milk spots " of the diffuse type which were larger than usual—up to 5 mm. in diameter—and raised above the surface. Some of them showed a haemorrhagic centre. There were also numerous greyish, ill-defined, older lesions. There were many petechiae in the lungs and a few small areas of consolidation. There were no adult *Ascaris* in the small intestine. Extraction of the liver failed to yield any larvae but a few were recovered from extractions of the lung and contents of the small intestine.

Both old and recent lesions were seen in sections of the liver, and one of the recent foci showed a parasite in cross section. The large size of the recent lesions was due to parenchyma destruction involving several lobules. In the lungs there were perivascular collections of eosinophils but the

bronchial walls were unaffected. The consolidated areas showed non-parasitic bronchopneumonia.

Pig 2737.—Control normal pig, killed at the same time as the preceding. The eosinophile counts were: 3·6 per cent. 26 days before slaughter, 2·9 per cent. 14 days later, and 3·0 per cent. just before the animal was killed. The liver showed a single whitish focus, 3 mm. in diameter, on the posterior surface and three indistinct diffuse lesions. The inferior portion of both lungs was consolidated. The faeces were negative for *Ascaris* eggs but contained strongyle eggs and forage mites. There was one adult *Ascaris* in the small intestine. The lungs, liver, and small intestine extractions yielded no larvae.

The single liver nodule was composed of lymphoid tissue with numerous " follicles," and a central area of necrosis with calcium deposition (silver nitrate test) and a number of multinucleate giant cells. The indistinct lesions proved to be old " milk spots " and the centre of one of them contained a hyaline pink structure, enclosed by lymphoid cells, and probably representing a degenerated parasite. The lungs showed non-parasitic bronchopneumonic areas.

Discussion of Results

This experiment confirmed the findings of Experiment 1. Unfortunately it proved impossible, by faecal examination of the sows, to select pigs free from *Ascaris* and so both experiments were in some ways confused by natural infestation. The effects of feeding 250,000 embryonated eggs were, however, so obvious as to leave no doubt that the liver lesions were the direct result. Occasional older lesions of the same type were clearly due to the natural infestation which did not unduly interfere with the experiment. The Baermann extraction method was not entirely satisfactory, in spite of the later modification of using large metal funnels and placing them in the hot room for several hours. It is probable that some tissue contained larvae and failed to disclose them by this method. The finding of larvae after feeding eggs, however, was regarded as presumptive evidence of migration.

It has been noted by previous workers that by giving a single large dose of eggs it is difficult, experimentally, to induce a heavy infestation with adult intestinal *Ascaris* and it seems that the giving of repeated small doses, as would occur under natural conditions, is more likely to be effective because it would seem that pigs acquire only a very slight immunity to reinfestation. The proportion of larvae which reach the liver and lungs, migrate up the trachea and finally grow to maturity in the small intestine in single dose infestations is very small when compared with the total number of embryonated eggs ingested. This proportion depends partly on the age of the host; for it is generally stated that pigs can be more easily infected during at least their first five months of life, and there is some slight evidence of natural resistance in older pigs. The vitamin A level of the diet may influence natural resistance, although most investigators have failed to demonstrate such an effect. We

consider it doubtful, however, whether this resistance extends to checking the ability to migrate to the liver since we have frequently found typical " milk spots " in bacon pigs at the abattoir and many such lesions were clearly of recent origin. This was an observation which, early in the investigation of the naturally occurring condition, suggested that the cause might not be *Ascaris*. It is possible, however, that migration to the liver and perhaps even the lung may take place at any age but that the ability of the larvae which reach the small intestine to grow to maturity diminishes with age and becomes completely lost in adult life. This aspect would seem to merit further study.

Another point requiring further investigation is the effect of giving repeated doses of *Ascaris* eggs to pigs, on subsequent reinfection. Our experience with pigs 2735 and 2740 suggests that fewer larvae migrate after repeated feeding at weekly intervals but a complicating factor was that the animals were considerably older at the end of the series of doses and no other animals were available to receive a single comparable dose at the same age. It is possible that both natural resistance and resistance to reinfection were operative in these cases.

BLOOD PICTURE IN PIGS FED WITH *Ascaris* EGGS

Some degree of polychromasia and the presence of occasional normoblasts are recognised as normal features in the pig. The only significant change observed in blood films prepared from pigs at varying intervals after dosing with *Ascaris* eggs was eosinophilia. The eosinophile count in the control pigs and in pigs at the time of feeding *Ascaris* eggs varied from 0·4 to 4·0 per cent., the mean value for 17 observations on 15 pigs being 2·3 per cent. Table I shows the values for individual pigs and Fig. 13 illustrates the eosinophilia which developed in the two animals receiving repeated doses at weekly intervals.

Animals given a single large dose of eggs showed an eosinophilia which was already evident in one animal after 24 hours (Pig. 2736) and reached its maximum at 10 to 20 days. The highest level, 23·7 per cent., was found in Pig 2739 S, killed ten days after receiving 250,000 eggs. In Pig 2742, killed 18 hours after a single large dose, not a single eosinophile was found amongst 2,000 leucocytes, and it may be that the blood eosinophils had been withdrawn to the site of migration—very large numbers were seen in sections of the mesenteric lymph nodes—and the bone marrow had not yet had time to respond to the demand for more.

The development and time relations of the eosinophilia in the pigs given repeated small doses of eggs (Fig. 13) are similar to those in animals given a single large dose and it appears that only the initial dose causes a marked rise in eosinophils in the circulating blood, the level returning to normal after four to five weeks. In the case of Pig 2735 the eosinophile count was 0·0 per cent. (1,000 leucocytes)

at the end of seven weeks, after seven weekly doses. The final rise to 4·4 per cent. in Pig 2740 when killed six days after a single large dose, having previously received ten small weekly doses, suggests that such a dose may produce a rise in eosinophils although small doses have no effect. It is of interest that the lesions in both these pigs showed heavy eosinophile infiltration, which was observed in the old " milk spot " lesions caused by the early doses as well as in the recent haemorrhagic liver lesions caused by the migration of *Ascaris* larvae a few days before slaughter.

Except during the initial migratory phase, a differential leucocyte count is therefore not likely to be of value in the diagnosis of *Ascaris* infestation in the pig. The graphs in the monograph by Roberts (1934) show essentially the same picture as described above, following either single or repeated doses of eggs.

GENERAL REMARKS

It is not suggested that these experiments contribute any additional information regarding the migration of *Ascaris lumbricoides* larvae in the pig. This has been studied very thoroughly by other workers and both Roberts in Australia and Schwartz in the United States of America have drawn attention to the liver damage which is produced during migration. In Britain, however, it is not generally agreed that " milk spot " is caused by migrating parasites, opinion amongst experienced veterinarians being divided (see White, 1941) as to whether parasitic or nutritional factors are responsible. Some degree of " white spot liver " is met with in about 10 per cent. of all pig livers at abattoirs in this country. We have previously tried to show that the lesions, in natural cases, are almost certainly due to migrating *Ascaris* larvae (White, 1941) and have now further shown, in this series of experiments, that lesions identical with the various types found in the naturally occurring condition can be produced by feeding embryonated *Ascaris* eggs. Our work leaves no doubt that the aetiology of " white spot liver " is explained by the migration of *Ascaris* larvae. A more widespread adoption of methods designed to control *Ascaris* infestation would do much therefore to reduce the incidence of the condition.

It would appear that some degree of *Ascaris* infestation is almost ubiquitous in this country as evidenced by the frequency of " milk spots." Not all pigs with liver lesions, however, show intestinal *Ascaris*, so that the actual incidence of migration is much higher than is to be inferred from figures based on the finding of adult ascarids in the intestine.

In addition to lung damage by migrating roundworm larvae there is the danger of secondary bacterial pneumonia. Although none of our experimental animals showed untoward symptoms after a single dose of 250,000 eggs it should be stressed that the pigs were kept out of doors in large runs and were well fed and

so probably possessed a fairly high resistance. Also, the experiments were carried out during the summer months; during the winter and in a poor environment it is probable that severe effects might have followed.

Control methods are very often based on a knowledge of the life history of the parasite. Of the pig *Ascaris*, we have a fairly detailed knowledge of the life history. A widespread adoption of, and strict attention to, methods of control compatible with practical pig husbandry to reduce *Ascaris* infestation have every chance of success. Not only would they reduce *Ascaris* infestation but these same measures would also reduce the incidence of other helminth parasites of the pig, generally improve the animals' health, and do much to eliminate wastage through mortality, unthriftiness, and, as this study has shown, condemnation of affected livers and their loss as food material.

SUMMARY

After feeding 20 pigs, eight to eleven weeks old, with from 200,000 to 400,000 embryonated *Ascaris lumbricoides* eggs, lesions were produced identical with the various types found in the naturally occurring condition of " milk spot liver " (chronic focal interstitial hepatitis). The aetiology of this condition, both natural and experimental, can therefore be ascribed to the migration of *Ascaris* larvae in the liver. The eosinophilia which followed single and repeated doses of eggs was studied. It is emphasised that a wider adoption of, and stricter attention to, control methods against *Ascaris* would successfully reduce the losses which ensue from roundworm infestation, not the least of which is the loss, as human food, of condemned " milk spot livers."

ACKNOWLEDGMENT

Our thanks are due to Mr. Macleod for preparing the photomicrographs.

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FIG. 1. Pig 2733 (Experiment 2), killed four days after feeding. Anterior surface of liver showing numerous lesions, some of which are depressed below the surface due to destruction of liver parenchyma. Approx. $\frac{1}{4}$ nat. size.

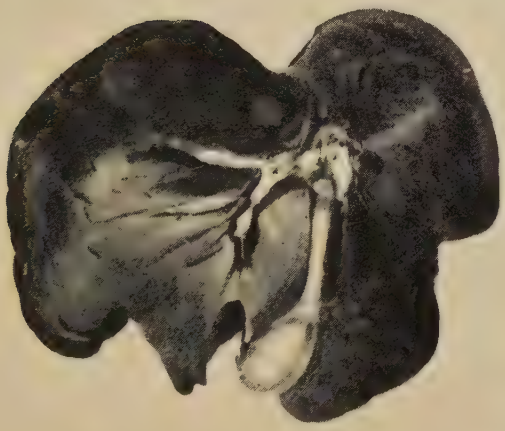

FIG. 2. Pig 2739 S (Experiment 2), killed ten days after feeding. Posterior surface of liver with numerous lesions, mostly of the diffuse type. Approx. $\frac{1}{4}$ nat. size.

FIG. 3. Pig 2734 (Experiment 2), killed 30 days after feeding. Posterior surface of liver showing lesions, some having an opaque central area. Approx. $\frac{1}{4}$ nat. size.

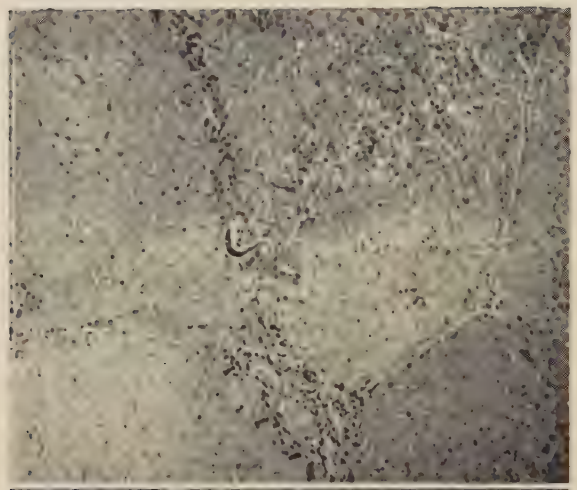

FIG. 4. Pig 2269 (Experiment 1), killed three days after receiving approximately 400,000 eggs. Section of liver showing a parasite in longitudinal section. The portal tracts are infiltrated with eosinophils and there is a track of destroyed liver tissue running from the parasite towards the top right of the field. H. & E. × 85.

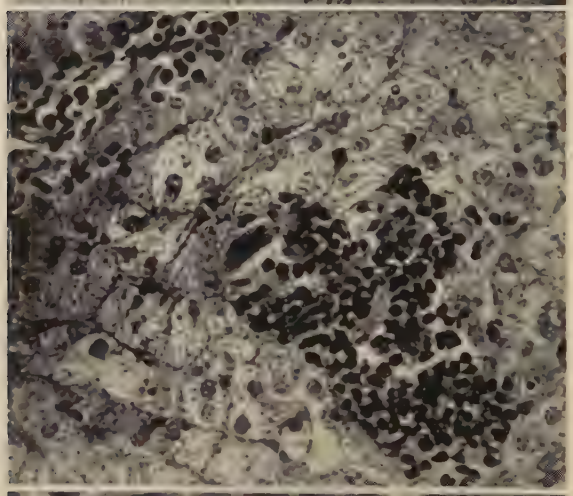

FIG. 5. Pig 2241 (Experiment 1), killed 48 hours after receiving 200,000 eggs. Oblique section through a parasite (just left of centre) seen near the periphery of a liver lobule. Eosinophils are seen in the portal tract (top left) and in the track of the parasite.
H. & E. × 1150

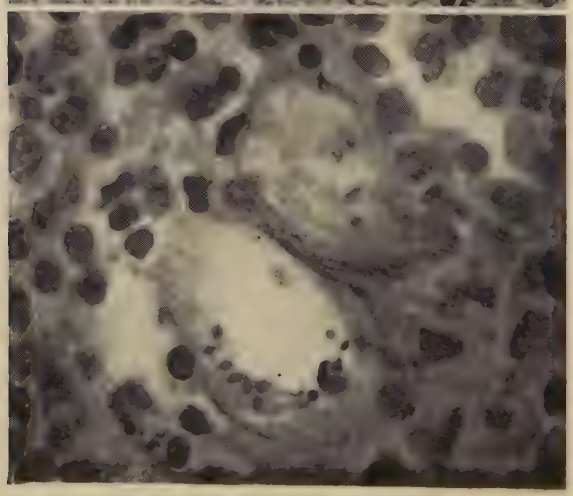

FIG. 6. Pig 2739 L (as Fig. 10). High magnification of part of a superficial lymphoid nodule in the liver, showing a parasite cut twice in cross section and surrounded by a fibrillary material. Cells surrounding the parasites were all mononuclears, and eosinophils were confined to the periphery of the lesion. The parasite shows the small blue staining particles, about 1 to 2μ in diameter, which are a constant feature. H. & E. × 1150.

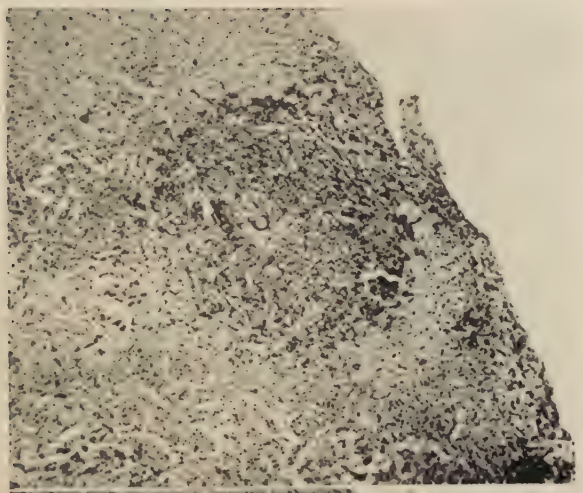

FIG. 7. Pig 2739 S (Experiment 2), killed ten days after receiving 250,000 eggs. Superficial liver lesion with a parasite in its centre, surrounded by lymphoid cells and a wide zone of eosinophils. The overlying liver capsule is heavily infiltrated with eosinophils.
H. & E. × 85.

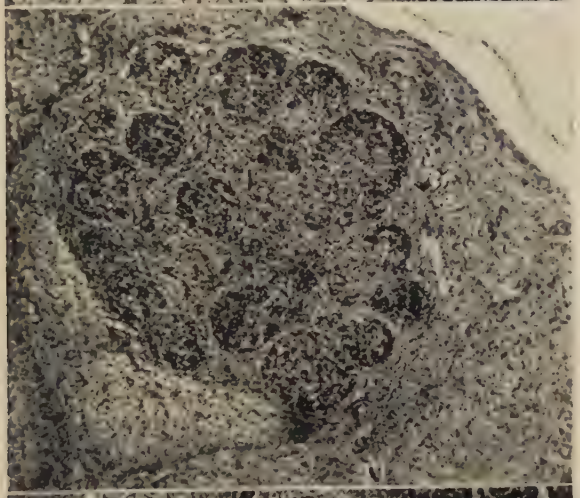

FIG. 8. Pig 2268 (Experiment 1), killed 27 days after receiving approximately 400,000 eggs. Section of liver showing a lymphoid nodule with well developed "follicular" pattern, replacing the greater part of a liver lobule. H. & E. × 56.

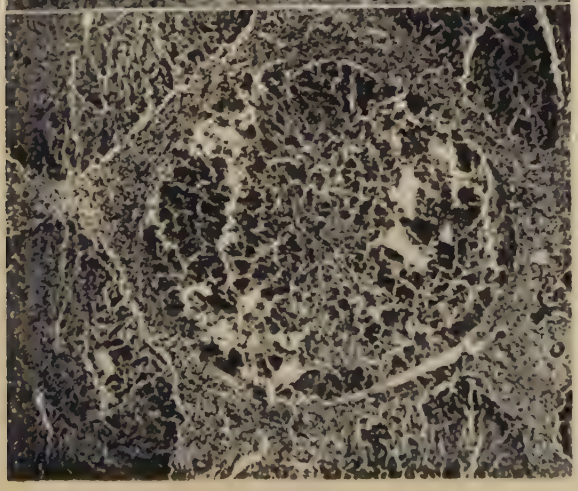

FIG. 9. Pig 2236 (Experiment 1), killed six days after receiving 400,000 eggs. Section of liver showing a deep haemorrhagic focus causing complete destruction of a liver lobule which is occupied by cellular debris, free red cells and masses of eosinophils. The surrounding septa are heavily infiltrated with eosinophils. H. & E. × 40.

FIG. 10. Pig 2739 L (Experiment 2), killed 20 days after receiving 250,000 eggs. Section of liver with superficial diffuse "milk spot" composed of interlobular septa greatly thickened by fibrosis and eosinophile infiltration. H. & E. × 40.

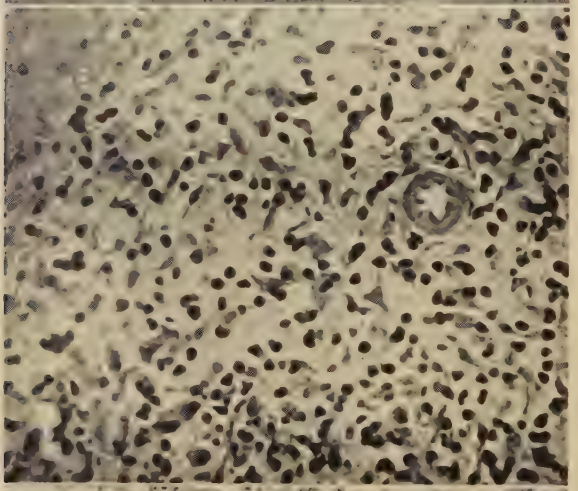

FIG. 11. Pig 2270 (Experiment 1), killed six days after receiving 400,000 eggs. Section through a portal tract showing fibroblast proliferation. The darkly stained cells are eosinophils. H. & E. × 300.

FIG. 12. Pig 2739 S (as Fig. 7). Section of lung showing perivascular and peribronchial accumulations of eosinophils. The alveoli near the centre of the field contain free red blood cells and eosinophils. H. & E. × 75.

TABLE I

No. of Pig	*No. of Eggs Fed*	*Period of Infestation*	*Eosinophils in Blood per cent. (1,000 leucocytes)*	
			Initial	*Final*
2742	250,000	18½ hours	1·9	0·0†
2736	250,000	24 hours	1·7	4·9
2241	200,000	2 days	1·3	4·7
2269	400,000	3 „	*	6·9
2733	250,000	4 „	2·6	10·6
2236	400,000	6 „	*	3·4
2270	400,000	6 „	*	7·1
2738	250,000	6 „	1·4	4·5
2739 S	250,000	10 „	3·0	23·7
2273	400,000	13 „	*	15·7
2739 L	250,000	20 „	1·4	12·6
2268	400,000	27 „	*	2·0
2734	250,000	30 „	3·4	7·2
2235	Control		*	1·6
2271	Control		*	2·2
2272	Control		*	2·4
2737	Control		3·6	3·0
2741	Control		*	4·0
2735‡	10×25,000		2·4	2·7
2740‡	10×25,000 + 250,000		0·6	4·4

* Signifies not examined. † No eosinophils in 2,000 leucocytes. ‡ See Fig. 13.

FIG. 13

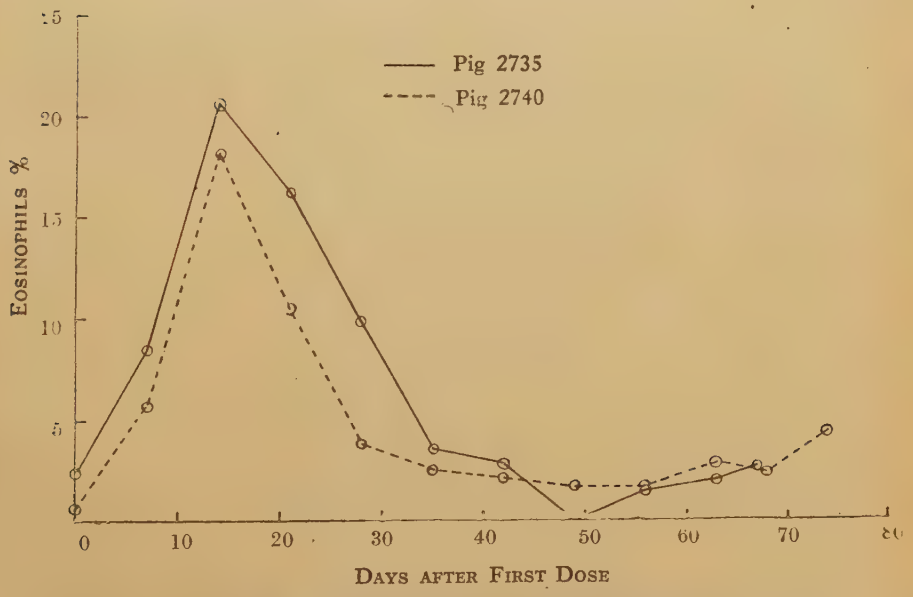

FIG. 13. Eosinophil counts of Pigs 2735 and 2740 (Experiment 2). Both pigs received ten weekly doses of 25,000 *Ascaris* eggs each, and Pig 2735 was killed four days after the last dose. Pig 2740 received an additional single dose of 250,000 eggs and was killed six days later.

URINARY CALCULI IN THE DOG, WITH ESPECIAL REFERENCE TO CYSTINE STONES

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INTRODUCTION

Urinary calculi are relatively common in the dog and the operations of urethrotomy in the male and cystotomy in the female are frequently carried out for their removal. Recurrence following removal is common in spite of regulation of the diet and various forms of medicinal treatment. Post-operative measures to prevent recurrence are likely to be of greater value if the composition of the calculi is known, since stones of different composition require different treatment. For example, rendering the urine more acid may be of value in preventing recurrence of phosphatic calculi but would probably render more likely the formation of cystine stones in dogs with cystinuria. During recent years it has been realised by medical urologists that a knowledge of the composition of calculi is essential if rational measures are to be adopted to reduce the likelihood of their recurrence.

We have carried out a rough qualitative analysis of a series of urinary calculi from dogs in an attempt to determine the relative incidence of the various types of stone and the characteristic features of each. The method of analysis used detected only the main constituents and gave only an approximate idea of their relative proportions, but the results show it to be sufficiently accurate whilst possessing the advantage of taking little time and requiring few reagents.

A series of 103 calculi—vesical, urethral and renal—has been examined by a combination of the methods recommended in various textbooks, the scheme for qualitative analysis of urinary calculi described by Domanski (1937), and certain tests included in the more detailed method of analysis suggested by Higgins and Mendenhall (1939).

METHOD OF ANALYSIS

1. *Description of Calculi and Physical Features.*—Number of stones, shape, size, weight and surface features. The calculi are cut or sawn in half and the cut surface examined for radial or concentric lamination, central " nucleus," variations of colour and consistence, and odour.

2. *Heating on a Platinum Spade.*—This preliminary procedure has been found extremely useful. Stones which burn away almost completely with a bluish flame and evolve a pungent odour of burning hair were found to consist of cystine. The presence or absence of fusion was noted and the proportion of the powdered calculus which burned away. Fusion, without loss by burning away, and a greyish black brittle residue was a feature of phosphate stones. Gentle heating of oxalate stones resulted in formation of carbonate which produced carbon dioxide when the heated stone was treated with dilute hydrochloric acid, similar treatment of the original stone

producing no evolution of gas. Further strong heating of oxalate stones gave a fine, white, soft powder which when moistened was strongly alkaline to litmus. Calculi composed mainly of urate or cystine left almost no residue.

3. *Chemical Tests.*—These were carried out with the powder resulting from sawing large stones in half or, in the case of small stones, by scraping with a scalpel so as to include portions representative of the whole stone. When the central and peripheral portions were different in appearance they were examined separately.

(*a*) *Murexide Test.*—One or two drops of concentrated nitric acid are added to a little of the powdered stone and evaporated to dryness on the water-bath. A pink or yellowish residue remains which gives a characteristic purple colour on addition of a drop of dilute ammonia. Uric acid stones gave a very intense colour with this test, whereas the trace of urate in the centre of a large proportion of phosphatic stones gave only a faint pink tinge. Xanthine gives a yellow residue with the murexide test, the colour changing to red with concentrated caustic soda. No xanthine was detected in any of the series.

(*b*) *Tests for Cystine.*—If heating on platinum suggested the presence of cystine a little of the powdered stone was dissolved in a few drops of ammonia, filtered, and the filtrate allowed to evaporate on a microscope slide. Cystine crystallises as regular hexagons within a few minutes. Further confirmation was obtained by heating the powdered stone with concentrated caustic potash solution and adding a few drops of a saturated solution of lead acetate: a brown or black precipitate of lead sulphide indicates the presence of sulphur. In addition, every stone in the series was tested for cystine by the spot plate test recommended by Higgins and Mendenhall (1939). A little powdered stone is placed in the depression of a spot plate and one drop each of 20 per cent. ammonia and 5 per cent. sodium cyanide added. After leaving for five minutes a drop of freshly prepared 5 per cent. sodium nitroprusside is added. A deep purple colour indicates cystine. The sensitivity of this test was examined by using pure cystine and also cystine mixed with calcium carbonate in concentrations which gave 10 per cent., 1 per cent. and 0·1 per cent. cystine in the mixture. About 10 mg. of substance was used for the test, this amount representing the "knife point" of powdered stone employed. The test was positive with a 1 per cent. mixture of cystine but a 0·1 per cent. mixture gave only an orange colour. It would thus appear that the test will detect 1 per cent. of cystine in calculi under these conditions.

(*c*) *Phosphates.*—To a little powdered stone in a test tube are added a few drops of a 10 per cent. solution of ammonium molybdate and an equal amount of concentrated nitric acid. Phosphate stones give a yellow colour in the cold and a massive yellow precipitate on heating. Calculi containing only a trace of phosphate give a yellow colour which is obvious only on heating.

(*d*) *Treatment with Dilute Hydrochloric Acid.*—To some of the powdered stone add about 5 ml. of 10 per cent. hydrochloric acid, warm to dissolve, and filter. Evolution of gas bubbles in the cold suggests the presence of carbonate, and this can be confirmed by testing for carbon dioxide by covering the mouth of the test tube (preferably a short wide one) with an inverted slide on which is a drop of filtered barium hydroxide solution. The drop, protected from the air, becomes cloudy in the presence of carbon dioxide.

The solution of the stone in hydrochloric acid is neutralised by adding 20 per cent. ammonia drop by drop until just alkaline to litmus. Oxalate or phosphate is precipitated, either as a turbidity or in crystalline form, depending on the cations associated with the acid radicle. A solution of 5 per cent. acetic acid is then added drop by drop until the mixture is just acid to litmus. Triple phosphate completely disappears with this adjustment of the *p*H, whereas stellar phosphates persist. To about half of the fluid add excess of acetic acid: phosphate dissolves and oxalate persists, but the latter will dissolve on adding a few drops of dilute hydrochloric acid. The other portion of the solution of the stone is allowed to stand for at least 30 minutes and the deposit examined under the microscope. Triple phosphate is seen as characteristic "knife rests," "coffin lids," etc., and stellar phosphates as fine feathery crystals. If no phosphate crystals are seen in cases where the molybdate test was positive this is because triple phosphate has gone into solution following acidification with acetic acid and in such cases the addition of a drop of ammonia to make the solution just alkaline to litmus will give triple phosphate crystals. Oxalate is seen as octahedral "envelope" crystals, often visible only under the 1/6th objective.

The presence of ammonia in the original stone can be identified by heating a little of it in a crucible with a few drops of concentrated caustic potash and covering the crucible with a filter paper on which are two drops of Nessler's reagent, one in the centre of the paper and the other beyond the edge of the crucible. Evolution of ammonia causes the central spot to turn brown before the peripheral one, the control spot being necessary on account of ammonia in the air.

The recognition of typical "envelope" crystals in calculi containing oxalate proved difficult, especially when this substance was present in large amounts. In such cases the precipitate resulting from neutralisation with ammonia was granular and crystals were sometimes obtained only if acidification was carried out as far as *p*H 4 to 4·5, so that crystallisation of oxalate occurred slowly.

The presence of oxalate can be confirmed by adding to the solution of the stone in hydrochloric acid a few drops of dilute sulphuric acid, warming to about 60° C., and adding 0·01 N. potassium permanganate drop by drop until a pink colour just persists. A few drops of solutions of oxalate stones usually decolorised about 20 drops of permanganate, whereas stones which proved by other tests to be free from oxalate rarely decolorised more than three or four drops. This test is of no value in detecting oxalate, however, if cystine or other organic substances are present in the original stone.

A final confirmatory test for oxalate can be made by mixing a little powdered stone in a spot plate depression with an equal amount of resorcinol (A.R.) and adding a drop of concentrated sulphuric acid. The appearance after a few minutes of a blue or greenish blue colour indicates oxalate and the reaction is usually most intense after about 30 to 40 minutes and has faded after two hours. The sensitivity of the test was determined by using pure potassium oxalate and 10 per cent., 1 per cent. and 0·1 per cent. mixture of oxalate with disodium hydrogen phosphate. Using 10 mg. of substance, the test was positive with the 1 per cent. mixture but no reaction was obtained with 0·1 per cent. oxalate. It is thus probable that this test will detect 1 per cent. of oxalate in calculi.

This method of qualitative analysis was found to yield consistent results and gave an approximate analysis of the calculi in a minimum of time, in most cases taking about 40 minutes. As most of the calculi in the series consisted mainly of a single chemical substance with other substances present in only small amounts, the method gave results sufficiently accurate for classification of the series.

RESULTS

Calculi from 103 dogs were examined and a summary of their situation, composition and the sex incidence is given in Table I. Chemical constituents which were present in only small amounts are given in brackets. As mentioned earlier, it is unlikely that the tests used would detect less than 1 per cent. of either oxalate or cystine.

Some of the more common types of calculi are illustrated in Figs. 1 and 2, and it was found possible in most cases to identify the main constituent of calculi from their gross characters and cut surface, together with the behaviour of the powdered stone when heated on platinum. The latter procedure is in our opinion of considerable value and was invariably included in the older schemes of analysis for calculi.

Vesical Calculi.—There were 70 cases of vesical calculi, in 65 of which the stones were confined to the bladder, whereas two were accompanied by urethral, two by renal and one by both urethral and renal stones.

Of the 69 cases of vesical calculi, 37 showed multiple stones and the remainder single calculi. The greatest number of stones in our own series of cases was 345, these being smooth, pyramidal stones composed of phosphates. Through the kindness of Major H. Kirk, M.R.C.V.S., we were able to examine one of the 1,530 vesical stones removed at operation from a dog and described in his book (Kirk, 1939). The stone consisted of phosphate. The largest single vesical calculus measured 70 × 55 × 40 mm. and weighed 160 g. (Fig. 2, No. 14). It was from a male Dalmatian and was composed of urate and phosphate.

An interesting finding was a mass of brownish " gravel " in the bladder of a Dalmatian (Fig. 1, No. 1), composed of myriads of small particles resembling sand as well as a few calculi measuring 2 to 3 mm. They were composed of urate.

An unusual type of vesical calculus is illustrated in Fig. 1, No. 12. There were several whitish stones with a nodular appearance somewhat resembling the crown of molar teeth. In the whole series there are only two examples of multiple calculi of this shape, both from Worcestershire, and they are composed of phosphates. Another unusual type of multiple vesical stones (Fig. 1, No. 11) consisted of spherical calculi with small sharp projections and composed of phosphates.

Urethral Calculi.—Among the 35 cases of urethral calculi, 22

TABLE I
COMPOSITION OF URINARY CALCULI FROM 103 DOGS

Vesical	*Urethral*	*Vesical and Urethral*	*Vesical, Urethral and Renal*	*Urethral and Renal*	*Vesical and Renal*	*Renal*
65 animals (35 ♀, 5 ♂, 25 not known)	31 animals (31 ♂)	2 animals (2 ♂)	1 animal (♂)	1 animal (♂)	2 animals (2 ♀)	1 animal (♀)
Phosphate 31	Cystine 15	Oxalate and phosphate 1	Urate 1	Oxalate 1	Phosphate (urate) 1	Urate (phosphate) 1
Phosphate (urate) ... 22	Cystine and phosphate 2	Oxalate ... 1			Phosphate and oxalate 1	
Phosphate (oxalate) 1	Phosphate 4					
Phosphate (carbonate) 2	Phosphate (urate) ... 3					
Oxalate 2	Oxalate 2					
Oxalate (phosphate) 2	Oxalate (phosphate) ... 2					
Urate 1	Urate 3					
Urate and phosphate 2						
Cystine 1						
Cystine and oxalate 1						

TABLE II
RECURRENCE OF CALCULI

Breed and Sex	*First Operation*			*Second Operation*		
	Age	*Site*	*Composition*	*Age*	*Site*	*Composition*
Dalmatian, ♂	2 years	Urethra	*	5 years	Urethra	Urate and phosphate
Dalmatian, ♂	*	Urethra	*	2 years later	Urethra	Urate
Pekinese, ♀	*	Bladder	Phosphate	6 months later	Bladder	Phosphate
Mongrel, ♀	7 years	Bladder	*	9 years	Bladder	Phosphate
Wire Hair Fox Terrier, ♂ . .	3 years 9 months	Urethra	Oxalate and phosphate	4 years	Urethra	Oxalate and phosphate
Cairn, ♀	7 years 6 months	Bladder	*	8 years	Bladder	Phosphate (urate)
Cross-bred Schipperke, ♂ ...	5 years 3 months	Urethra	*	5 years 6 months	Urethra	Cystine
*, ♂	*	Bladder	Oxalate	1 month later	Urethra	Oxalate

* Not known.

were of multiple stones, but this figure is not necessarily accurate as it would be easy for one or more of the small urethral calculi to be missed at operation. All the urethral stones were small, varying from a tenth of a millimetre up to 6 mm., they were usually spherical, and the surface was smooth and cream coloured except in a few cases where there were smooth nodular projections (" Mulberry stones ").

Recurrence.—Details are given in Table II of eight dogs in which urinary calculi removed at operation were reported as having recurred. It is certain that the incidence of recurrence is higher than is indicated by this figure, but information as to the subsequent history of most of the animals in the series is lacking. The interval between operative removal of calculi and symptoms necessitating a second operation varied from one month to three years and in the three dogs from which both stones were analysed it will be seen that the same type recurred.

Breed and Age Incidence.—Conclusions as to the incidence of urinary calculi in different breeds can be drawn only from a large series of cases, and then only by comparison with the relative numbers of different breeds treated by veterinarians for conditions other than urinary lithiasis. If this is not done a high incidence in any given breed may be due solely to the greater popularity of that breed. The incidence in the various breeds in the present series is : Pekinese (nine), Mongrel and Scottish Terrier (eight each), Wire Hair Fox Terrier (seven), Dalmatian and Spaniel (five each), Welsh Corgi, Bulldog, Cairn and Terrier (three each), Airedale and Sealyham (two each), Great Dane, Setter, West Highland Terrier, Retriever and Old English Sheepdog (one each).

Of 40 dogs with urinary calculi for which the exact age was known, 25 were between four and eight years old, the youngest was two years and the oldest 18 years. It may be that the low incidence in old dogs is explained by recurrence of calculi resulting eventually in fatal urinary obstruction or in destruction of the animal. Here, too, it is unwise to draw definite conclusions without examining a larger series of cases and considering all the possible factors concerned.

INDIVIDUAL TYPES OF CALCULUS

1. *Phosphate Stones.*—These are by far the commonest type of vesical calculus and are generally considered to be secondary to cystitis. They are either single, with a smooth or rough surface, or they are multiple with smooth surfaces and an irregularly pyramidal shape. The central portion of many of these calculi contains a trace of urate. The cut surface is white and crystalline, sometimes with radial or concentric lamination, and the consistence of the large single stones is usually like that of a stick of chalk.

Measures to prevent recurrence of phosphate stones in man include acidification of the urine by the use of a ketogenic acid ash diet and administration of ammonium chloride and ammonium

nitrate. In view of the association of phosphatic calculi with infection of the urinary tract a diet rich in vitamin A is generally recommended.

2. *Oxalate Stones.*—There were six cases of pure oxalate stones —two vesical, two urethral, one vesical and urethral, and one renal and urethral. Vesical oxalate stones had an uneven surface with sharp projecting crystalline plates and were usually bloodstained. They were extremely hard and cut like lump sugar (cf. phosphate stones). Urethral calculi composed of oxalate were multiple, very hard and roughly spherical. They could be distinguished from cystine stones by their hardness, their white rather than cream colour, and the absence of a waxy feel.

Although much of the oxalate excreted in the urine comes from the food, it is probable that some is endogenous. In man it is usually advised that the diet should contain a minimum of foods rich in oxalate. Barrett (1942) reports that foods rich in oxalate, such as rhubarb and spinach, fail to cause a rise in urinary oxalate if consumed mixed with milk, as insoluble calcium oxalate is formed and oxalate absorption prevented. Administration of drugs to alter the reaction of the urine is unlikely to have much effect in preventing recurrence since oxalate is precipitated over a wide range of *p*H : acidification of the urine is contraindicated as it would favour precipitation.

3. *Urate Stones.*—No attempt was made to distinguish between uric acid and urate, although the evolution of brown fumes when the powdered stone is treated with concentrated nitric acid probably indicates the presence of ammonium urate and this occurred with all the uratic calculi in the series. The 26 phosphatic calculi which contained a trace of urate showed the typical features of phosphate stones. Of the seven animals with urate stones for which the breed was known, four were Dalmatians and there was one case each in an Airedale, Bulldog and Welsh Corgi. Urate stones were hard, brittle and usually concentrically laminated. They were usually of a brownish or greenish colour and when scraped they gave a fine brownish powder, like liquorice powder. The urethral stones had a smooth surface with a metallic lustre and the concentric laminae were like layers of eggshell.

Urate stones are encountered mainly in Dalmatians and little can be done to prevent their recurrence. Keller (1940) states that " high uric acid " is inherited in the Dalmatian as an almost completely recessive, non-sex-linked unit character, depending on the presence of a single pair of Mendelising genes. Attempts are being made to establish a line genetically free from " high uric acid."

4. *Cystine Stones.*—Cystine calculi have previously been considered rare in the dog and only a few cases have been recorded. We were therefore surprised to find 19 cases in which the calculi were composed mainly of this substance. They comprised two cases of vesical calculi and 17 of urethral calculi. One of the former

contained some oxalate and two of the latter some phosphate. The urethral stones were buff or cream coloured, rounded, smooth and sometimes had a nodular surface. They were not so hard as phosphate stones, and when scraped they yielded a white powder with a waxy feel. Cystine stones comprised 17 of the 31 cases of urethral calculi and they may thus represent the commonest type of stone necessitating urethrotomy in the male dog.

DISCUSSION OF CYSTINE STONES

The occurrence of cystine stones in the kidney and bladder of dogs is mentioned by Joest (1924). A new impetus was given to the study of cystinuria in the dog by the description of a cystine stone in an Irish Terrier by Morris and his co-authors in 1935. These workers later found the son of a male litter mate of the original dog to be cystinuric and by breeding this animal to related non-cystinuric females they obtained over 300 progeny of which 12 were cystinuric. In a recent paper Hess and Sullivan (1942) have studied two of these cystinuric dogs and have observed the effect of feeding cystine, cysteine and methionine at different protein levels. They were able to confirm the observations of earlier workers that feeding cystine had no effect on the amount of cystine excreted in the urine. Blamey and Brand (1939) recorded the occurrence of cystinuria in two Scottish Terriers, one of which had small vesical cystine stones. Sjöberg and Nilsson (1940) described multiple small urethral stones in an Irish Terrier which contained 91 per cent. of cystine. All the animals with cystine stones or cystinuria were males.

That cystinuria is not confined to any particular breed is shown by the fact that the eight animals with cystine stones in the present series for which the breed was known included Scottish Terrier (two), Bulldog (two), mongrel (two), Spaniel and crossbred Schipperke. Blamey and Brand (1939) refer to cystinuria in the Dachshund, Irish Terrier and Scottish Terrier.

It is well known that cystinuria in man is mainly confined to the male sex and the same appears to hold for the dog. All our own cases have been in male dogs. Vesical stones are generally recognised as uncommon in the male dog—five males to 35 females in the present series—whereas the narrow urethra of the male, and especially that portion of the urethra which passes through the groove in the os penis, is a common site for the lodgment of small calculi. In the bitch urethral obstruction is rare since quite large calculi can be passed through the short, wide, distensible urethra. If, as seems likely, cystinuria in the dog is predominantly a disorder of the male sex, this would explain why cystine stones form so high a proportion of urethral calculi in this species. If cystinuria occurs also in the bitch it is unlikely that small calculi would cause symptoms of urethral obstruction and thus, unless large stones were present causing urinary obstruction, the condition might not be recognised. In the case of male dogs from which urethral calculi are removed at operation

it is possible that in many cases small calculi are also present in the bladder but fail to cause symptoms in this situation.

It is difficult to explain why cystine stones have hitherto been considered rare in the dog. A possible explanation is that practitioners who have removed urinary calculi rarely submit for chemical analysis the small urethral stones but are more interested to know the nature of the large and more striking vesical calculi.

As in man, it is probable that only a proportion of cystinuric dogs would ultimately develop cystine stones and not all of these would develop urinary obstruction necessitating urethrotomy. The incidence of cystinuria in the dog may thus be considerably higher than is revealed by analysis of this series of calculi.

Some urologists have stated that cystine stones cannot be visualised by X-rays, but Morrison (1940) was able to detect them in 69 of 75 cases in man and Sjöberg and Nilsson (1940) report their demonstration by radiography in the urethra of a dog. Since cystine is readily soluble in alkaline solutions it is rational to attempt to keep the urine alkaline and this procedure has been reported to have brought about dissolution of cystine calculi in the renal pelvis in man and to have prevented their re-formation. In man a diet containing the minimal amount of protein consistent with health is advised and the fluid intake is increased so as to keep the cystine level in the urine below a concentration at which it is likely to crystallise out.

SUMMARY

A series of 103 cases of urinary calculi in dogs has been studied, the stones being analysed by a qualitative method capable of detecting the main constituents.

There were 65 cases of vesical calculi, 31 urethral, one renal, and the remaining six were in more than one part of the urinary tract.

ILLUSTRATIONS

FIG. 1

1.—" Gravel " and small calculi from bladder of 18-year-old Dalmatian, ♂. Urate.
2.—Vesical and renal calculi from four-year-old Bulldog, ♂. Urate.
3.—Six of nine urethral stones from two-year-old Bulldog, ♂. Cystine.
4.—Eight of 59 urethral stones from male dog. Cystine.
5.—(*a*) Two of three urethral stones from four-year-old Wire Hair Fox Terrier, ♂. Oxalate and phosphate.
(*b*) Vesical stone from the same dog three months later. Oxalate and phosphate.
6.—Six of 35 urethral stones from seven-year-old Pekinese, ♂. Phosphate.
7.—(*a*) Vesical calculus from dog, ♂. Oxalate.
(*b*) Four of eight urethral stones removed one month later. Oxalate.
8.—Seven of 28 urethral stones from seven-year-old cross-bred Retriever, ♂. Phosphate.
9.—Two calculi removed at autopsy from the urethra of a Dalmatian, ♂. Urate. Urethrotomy for urethral stones two years previously.
10.—Vesical calculus from eight-year-old Wire Hair Fox Terrier, ♂. Oxalate (phosphate).
11.—Three of four vesical stones from seven-year-old Sealyham, ♀. Phosphate.
12.—Five of 60 vesical stones from Great Dane, ♀. Phosphate.
13.—Renal calculi from three-year-old Airedale, ♂. Urate.

Fig. 1

18a 18b

FIG. 2.

Most of the calculi consisted almost entirely of one chemical constituent—phosphate, oxalate, urate or cystine—but in 38 cases a second substance was present, usually in small amount.

Vesical calculi were much more common in the female and most of them consisted of phosphate, often with a trace of urate in the centre.

Of the 31 cases of urethral calculi, all in male dogs, 19 were composed mainly of cystine and this substance also formed the main constituent of two cases of vesical stone.

The features of the different types of calculi are described and some general suggestions are made for preventing their recurrence after removal. Chemical analysis is a necessary preliminary to such treatment.

The high incidence of cystine calculi in dogs, all males, suggests that cystinuria is much commoner in this species than was formerly believed.

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ILLUSTRATIONS

FIG. 2

14.—Vesical calculus from five-year-old Dalmatian, ♂. Urate and phosphate. Previous history of catheterisation and urethrotomy.
15.—Vesical calculus from dog. Phosphate.
16.—(*a*) Vesical calculus from Pekinese, ♀. Phosphate.
(*b*) Second vesical stone removed six months later. Phosphate.
17.—Vesical stone from nine-year-old Mongrel, ♀. Phosphate (carbonate).
18.—(*a*) Five of 22 vesical stones from four-year-old Scottish Terrier, ♀. Phosphate (urate).
(*b*) Renal calculus from left renal pelvis of same dog found at autopsy three months later. Phosphate.
19.—Vesical calculi from nine-year-old Mongrel, ♀. Phosphate. Cystotomy for vesical stones two years previously.

STUDIES ON THE HAEMATOLOGY OF SHEEP

I.—THE BLOOD-PICTURE OF HEALTHY SHEEP

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INTRODUCTION

This work was undertaken to discover the clinical merit of blood examinations in sheep, and to ascertain whether or not any standards could be laid down that would add to the value of the sheep as an experimental animal. This article is limited to the blood-picture of healthy sheep. Some standards for sheep in the United Kingdom have already been set by Fraser (1930). Both Fraser and Wirth (1931) have summarised the results of workers in other countries.

The sheep used in our investigation were Scottish hill sheep—Cheviots and Blackfaces—and their crosses with the Border Leicester breed—Half-breds and Grey faces respectively. In addition, five sheep of the Suffolk breed were included. No special test for health was carried out, the sheep being assumed healthy in the absence of obvious signs of disease. With the exception of new-born lambs, it is probable that all sheep were to some extent infested with helminths. The work is based on samples obtained from 171 sheep, of which 22 were bled on several occasions. In Fig. 1 and Table I not more than one sample from any individual has been employed.

TECHNIQUE

Three criteria were used in selecting a suitable technique, *viz.* that the methods (1) should be applicable under field conditions; (2) should not be too tedious, if found practicable, to be repeated by other workers; (3) should be sufficiently accurate to indicate the distribution of the normal range.

Procuring Blood Samples.—All sheep were bled from the jugular vein. Fresh blood was used to make blood films and to fill coagulation tubes; oxalated blood was used for the remaining estimations.

Leucocytes.—For the leucocyte count blood was diluted to 1 : 20 with 4 per cent. acetic acid by means of a Piney pipette; cells lying in 2 sq. mm. in both chambers of a haemocytometer were counted; where the difference between the two sides exceeded 10 per cent. a further dilution was counted. For the differential count, films were stained by Leishman's stain and examined by Schilling's four field meander method. The frequent use of the classification of the cellular elements of blood as material for statistical analysis has created a desire for mathematical accuracy to an extent unheard of in the histological examination of other tissues and it has become quite common for pathologists responsible for routine differential counts to look askance at counts which they know to be under 400 or 500 cells, while in some veterinary investigations even 800 cells have been classified. As in this investigation only 200 cells have been classified, it is felt that this requires some justification. The advantage of counting a large number of

cells is the reduction of the sampling error. Thus where cells represent about 50 per cent. of the total present, the sampling error when 200 cells are counted exceeds seven cells per hundred only once in twenty times; where 400 cells are classified this error is reduced to five cells. To classify 200 cells may take from 20 to 60 minutes, depending on the film; to double this time by classifying another 200 cells on the chance of reducing a large error by two cells would be justified if there existed some condition in which this increased accuracy was vital; no such condition is revealed in textbooks on haematology. In the view of the writer the extra time spent on the examination of the film is out of all proportion to the additional clinical help derived from it, and the more extensive examination is akin to observing the respiration rate for 30 minutes and recording it correct to 0·1 of a respiration. The full error in the technique used will be referred to in a later article when dealing with the variation in individuals.

Leucocytes have been classified according to Schilling's haemogram. Most cells are easily recognised after reference to a textbook on histology or haematology, and no special description appears necessary. Difficulty may occur, however, in differentiating between atypical monocytes and atypical lymphocytes; points of distinction are given in textbooks but it is more important to have a clear mental impression of the typical cells. A point of discrimination used by the writer is that in lymphocytes the nucleus appears as a flat pancake-like body in which the chromatin is so disposed that it gives an impression of a wrinkled surface, while in the monocyte the nucleus appears lobular with an undulating surface, somewhat resembling an island with hills and valleys. In the few cases where cells have defied classification they have been classified as lymphocytes.

Neutrophils have been classified by Schilling's index, using the following criteria. Nuclei varying in appearance from a shape resembling a biscuit with a piece bitten out to a shape resembling a thick horseshoe with the heels parallel (see Osgood and Ashworth, 1937, cell No. 76) were classified as "Young forms"; nuclei varying from a thick horseshoe shape with the heels opened out to a simple ribbon-like nucleus shaped variously as a U, C, S or W (see Osgood and Ashworth, cell No. 77) were classified as "band forms"; and nuclei showing commencing lobulation (see Osgood and Ashworth, cells No. 78 and 79), or ragged in structure (Schilling's "Degenerative band forms") were classified together with all the segmented cells as adult neutrophils or polymorphs.

Erythrocytes.—For the red count, blood was diluted to 1 : 200 with saline by means of a Piney pipette; at least 250 cells were counted on both sides of the haemocytometer, and where the difference between the two sides exceeded 10 per cent. a further dilution was counted. The packed cell volume was obtained by spinning duplicate small haematocrit tubes for an hour. Haemoglobin content was estimated by a Zeiss Ikon haemometer, a modification of the Sahli method; with this instrument 30 estimations duplicated by the Biochemical Department using a Newcomer disc, showed that in 29 cases the Zeiss Ikon gave the higher reading, the increase averaging at 1·5 g. per 100 ml. Where Price-Jones curves were drawn up a modification of Martin and Hynes method was used and the class intervals varied by 0·5 micron. Fragility was tested by salt solutions varying by 0·05 per cent. NaCl and results were read after standing overnight, only the maximum haemolysis was noted. In view of the fact that the erythrocytes fell into only six classes by this method it would appear that a variation of 0·02 per cent. between solutions would be more suitable.

Thrombocytes.—These were counted unstained in the same preparation as the red count after this had been completed.

Properties of Whole Blood.—Coagulation time was estimated by a capillary tube method in which the tubes were immersed in water at 39° C. and tested each 30 secs. Viscosity was measured by Denning Watson viscosity tubes and the mean of two readings recorded. Specific gravity was estimated by using mixtures of benzol and chloroform of known densities; the method has been more fully described elsewhere (Stewart and Holman, 1940). Sedimentation was measured by using the apparatus described by Shufflebottom (1935), recording the fall in tubes roughly 7 mm. in external diameter after 24 hours.

GENERAL RESULTS

For simplicity in treatment the results have been divided into General Results and Variations from the general results. The general results are restricted to sheep sampled between the months of June and December inclusive; lambs born in March or April are therefore excluded and the lowest age represented is approximately three months. These general results are summarised in Fig. 1 and Table I.

For the results to be statistically valid it was necessary to include the variation due to error, and therefore even when complementary measurements or a subsequent estimation indicated that a large error had been made, the original estimation was included when calculating both the mean and the standard deviation. Marked abnormalities, either due to error or to a supposed abnormality in a sheep, have not, however, always been included in the diagram of the distribution, and in a few places single variates lying more than three standard deviations from the mean have been removed and the number of sheep and the maximum or minimum adjusted accordingly.

Leucocytic Picture.—The average leucocyte count, as seen in Fig. 1, was 9·2 thousand with a S.D. of 3·1. This is very similar to Fraser's figures of 8 thousand with a S.D. of 1·9 for ten sheep; in fact, it will be seen from Fig. 1 that the figure of 8 thousand represents the mid-point for the mode of our distribution, but that in our results the curve is somewhat skewed to the left, with two counts of 17 thousand (and one count of 28 thousand omitted from diagram) helping to increase the value of the average.

With regard to the different types of leucocytes, our ratio of neutrophils to lymphocytes average at 24 : 67 is compared with Fraser's ratio of 39 : 52. This shows a considerable difference and, although with one exception Fraser's figures for neutrophils fall within the distribution shown in Fig. 1, a statistical comparison shows a significant difference of the means with $t = 5{\cdot}64$ and $P =$ less than $0{\cdot}001$ where $n = 121$, and this will be referred to in the discussion. Fraser's mean figure of 3·2 for monocytes falls within our modal class, and his figures for eosinophils with a mean of 4·9 and a S.D. of 6·1 is in close agreement with our results.

Fourie (1931), working in South Africa on Haemonchus infestation in young sheep (exact age and breed not recorded), gives the results of blood examinations on eleven young sheep believed to be free from worms. Taking the first samples for each of his sheep, the following figures were obtained for leucocytes. The total leucocyte count showed an average of 8·4 thousand and a S.D. of 3·4; here the mean is again almost identical with the mid-point of the modal class, with the standard deviation in close agreement with our figure. The ratio of neutrophils to lymphocytes averages 33 : 62; this average for the neutrophils has a S.D. of 18·2 and therefore does not disagree with our result, while the range of 5 per cent. to 71 per cent. for eleven sheep gives a wider distribution than our range for 113 sheep, suggesting our range is not excessive for healthy animals. Fourie's proportion of monocytes, 2 ∓ 2, is close to ours and his proportion of eosinophils 2 ∓ 2·6, well within one standard deviation of our results.

The nuclear index has been found to show variation with age, and this is dealt with under the appropriate heading. The point has been made in discussing technique that neutrophil cells with nuclei of mature appearance were classified as polymorphs even when the nucleus was not segmented. In Table I we show the distribution of these cells among 15 four-month-old sheep and it can be seen that the variation in their distribution renders them useless as a sensitive clinical guide, particularly as 15 per cent. of the differential count may represent more than two-thirds of the total neutrophil proportion.

Erythrocytic Picture.—The erythrocyte count, with an average of 11·5 million per c.mm., is higher than Fraser's figure of 10·3 for ten sheep and lower than Fourie's figure of 12·4 for eleven young sheep. The standard deviations are 1·8, 1·4 and 1·6 respectively. This is a fairly close agreement, with the arithmetical mean of these two workers scarcely more than standard deviation from our own.

Our mean figure for haemoglobin was 12·4, with a standard deviation of 1·4, and in the section on technique it was noted that the instrument gave higher readings than a Newcomer disc used in the Biochemical Department. Unfortunately, both Fraser and Fourie give their results in percentages and no comparison is possible. Allcroft (1941), working in England and Wales, gives a mean of 11·5 for ewes and his figures give a standard deviation of 1·5. Wintrobe (1942), using four sheep in America, gives a mean of 12·9, and Wirth, quoting Welsch, records a range of 11·3 to 12·7. The fact that our haemometer gives a somewhat higher reading than another instrument and the close similarity between our standard deviation and that of Allcroft, suggests that there is little difference in the haemoglobin readings between sheep in Scotland and England or Wales. The mean corpuscular volume in our investigation averaged 27·4 cubic microns, compared with Fourie's figure which works out at 29·4, and Wintrobe's figure of 35 for four sheep; there

is thus a close agreement between our figures and those of Fourie, but Wintrobe's figure falls on the extremity of our distribution curve. Our mean figure for the mean corpuscular haemoglobin concentration is higher than Wintrobe's figure of 35 per cent.; in view of the absence of any information about the sheep he used, this has little importance.

Price-Jones curves were made for only three sheep, with the following results:—

No.	*Mean Diameter*	*Standard Deviation*	*Minimum Diameter*	*Maximum Diameter*
1	4·7	0·38	4·0	6·0
2	4·9	0·29	4·0	6·0
3	4·5	0·35	3·5	5·5

This is in close agreement with Chamberlin (1933), who gives the corpuscular diameter for Australian sheep as lying between 4 and 6 microns, and with Innes and Shearer (1940), who examined a smear from a pregnant ewe in England and found a mean diameter of 4·2 microns and a standard deviation of 0·48. The present tendency is to rely upon the packed cell volume for an estimation of corpuscular size, for, although the actual classification takes a pair of workers only about 25 minutes once the apparatus is set up, in laboratories where the microscope is continually required for other purposes, the process of fixing and calibrating the microscope in addition to the estimation makes the method tedious. Thus, as the Price-Jones curve offers little or no advantage over the estimation of the corpuscular volume, the method was not included in our routine examination.

Few workers have examined the erythrocytic fragility, and our results (Fig. 1) can be compared only to those of Kohanawa and Kadono, quoted by Wirth. These workers found a range of 0·45 to 0·57 per cent. NaCl for maximum haemolysis with the mean at 0·5. These results show the cells to be more resistant, but it is possible that this was due to their using young animals.

Thrombocytes.—Platelet counts on 29 sheep gave no indication of a normal curve (see Table I) and as diseases affecting the numbers of these bodies are of little clinical importance in farm animals, other than the dog and the horse, the technique was discontinued. Fraser, counting platelets in stained blood smears, gives a range of 250,000 to 750,000 per c.mm. (number of sheep and S.D. not stated) and a mean of 490,000. Wirth shows a wide difference among various workers.

Properties of Whole-Blood.—The figures for coagulation time in sheep are shown in Fig. 1. Owing to variations of method they cannot be compared directly with the results of other workers. Wirth quotes the following results. Marek, using a glass-tube method (method of Lee and White) at room temperature, gives a range of 4 to 8 minutes; Haag, using the same method in farm buildings during winter, gives a range of 3 to 5 minutes; Schubert,

testing blood at intervals with a needle, found traces of fibrin at between 1·25 and 1·5 minutes. When Burker's apparatus at 25° was used, Kuhar recorded 5·1 to 5·5 minutes and Amendt, 2·5 minutes.

Our results for the sedimentation rate, as assessed by the Shuffle-bottom apparatus, are shown in Table I.

TABLE I

SUPPLEMENTARY DISTRIBUTIONS

	Sedimentation Rate											
min.	1	2	3	4	5	6	7	8	9	10		
	1	19	12	6	7	1	3	1	0	1	50 sheep	
	Thrombocytes											
10^3. per c.mm.	75	125	175	225	275	325	375	425	475	525	575	
	1	3	3	7	3	2	2	3	1	1	2	29 sheep
	Differential Count. Neutrophils											
Per cent.	0	1	2	3	4	5						
Band forms	52	32	8	8	1	3	104 sheep					
Per cent.	2	4	6	8	10	12	14	16				
Adult unsegmented polymorphs	3	2	2	1	2	2	1	2	15 sheep			
	Absolute White Counts											
10^2. per c.mm.	7	12	17	22	27	32	37	42	47	52	57	
Neutrophils	6	21	34	19	9	10	4	1	2	5	2	113 sheep
10^2. per c.mm.	20	30	40	50	60	70	80	90	100			
Lymphocytes	3	9	23	21	25	16	8	5	2	112 sheep		
10^2. per c.mm.	0	0·75	2	3	4	5	6	7	8	9	10	11	12	13	14	
Monocytes	2	22	34	18	9	7	7	8	1	2	2	1	–	–	–	113 sheep
Eosinophils	13	27	11	17	11	8	8	4	5	2	2	2	0	2	3	115 sheep
Basophils	56	51	5	2	1	–	–	–	–	–	–	–	–	–	–	115 sheep
Band forms	52	34	10	3	3	2	–	–	–	–	–	–	–	–	–	104 sheep

Wirth quotes the results of an investigation by Zott, using undiluted blood in a capillary tube, recording a fall of 1 mm. in 2 hours and 6 mm. in 24 hours. Our experience with this test suggested that it was rather haphazard and that duplicate readings occasionally showed a big difference; further, where changes were marked, they were concomitant with a change in the erythrocyte count and hence more reliably interpreted by that method. The test appeared to be of little clinical value and was discontinued after preliminary observations.

The specific gravity of sheep's blood (Fig. 1) averaged 1052,

where water equals 1000; Bonard (Wirth) gives a range of 1040 to 1046, figures on the low side of our distribution curve, and Krugers a range of 1026 to 1027, figures well outside our normal range. The viscosity averaged 5·8 times that of water (Fig. 1). The only other figures we have for viscosity are from Fraser, who gives several estimations made with a Hess viscometer on three sheep; these figures range from 2·2 to 4·0, averaging around 3·0, the majority of his estimations thus being outside our distribution.

Wirth, discussing sedimentation rate, states that the correlation between this and the specific gravity or the viscosity in the different species is not yet known. Using scatter diagrams, we have attempted to investigate the existence of correlations. With estimations from 24 samples, it was impossible to suggest a relation between viscosity and sedimentation; with 50 samples no correlation was apparent between the red count and sedimentation; with 33 samples a rough correlation was observed between sedimentation rate and specific gravity, in that groups of samples gave the following averages: sedimentation rate 2 mm., S.G. 1056; sed. 4 mm., S.G. 1053; and sed. 7 mm., S.G. 1048.

Other correlations were sought, with negative results, between the following constituents. Specific gravity and viscosity with 75 samples; erythrocyte count and viscosity with 108 samples; and haemoglobin or packed cell volume and specific gravity with 88 samples in both cases. In every diagram, however, some of the extreme values coincided. For example, a sample with a haemoglobin very low or very high in the normal range would usually have a correspondingly low or high specific gravity, but with samples not at the extremes no suggestion of correlation was evident. This suggests that there is only a partial correlation between these attributes and that this is often obscured by other factors varying in individuals, or that the measurements are too distorted by error to reveal the correlation. The former view is supported in Fig. 2 by the apparent correlation between the erythrocyte count and its complements to the viscosity, where multiple samples have been taken from the same individuals, and also by the fact that, using 20 different samples from one individual, some correlation between the packed cell volume and the specific gravity was apparent, with a coefficient of correlation of 0·55, which is significant where $n = 18$ (Fisher, 1938. Table V.A.).

Thus, summing up, it can be seen that while correlations are apparent when multiple samples are taken from one individual, or a small group of individuals, these correlations are obscured when single samples from a number of individuals are used, and therefore the correlation between these attributes has little practical importance.

VARIATIONS

To give some indication of the variation due to "age or season," a group of four sheep was bled each month for two years. When

the first samples were taken the sheep were two or four weeks old.

Variations Due to Age

Erythrocytes.—From Fig. 2 it can be seen that the red count commenced with a low average of *c.* 9·2 million in May and again descended to this level during the following April; the highest level was reached during December of the second year. These changes are not believed to be due to age and will be considered under the heading of seasonal variations. The haemoglobin did not agree with the primary low values obtained for the red count for the first few months but thereafter was in close agreement. The packed cell volume showed a big contrast to the red count in the primary samples with the result that the M.C.V. showed a high level for the first month; after this the packed cell volume agreed fairly well with the red count, the M.C.V. varying only by three or four cubic microns. The M.C.H.C. showed surprisingly little variation and is practically a straight line.

These results suggest that although the red count was lower during the first two months of life, the haemoglobin concentration in the blood was kept normal by the larger size of the erythrocytes. The larger average cell size of young lambs where growth is rapid, would appear feasible in that macrocytes may be produced whenever haempoiesis is stimulated above a certain rate, but this point calls for confirmation.

By increasing the number of lambs under one month old to 15 (the maximum available) it was found that average cell volume for this group was 35·5 cubic microns as compared to the average of 27·4 given in Fig. 1 for sheep and lambs over three months. From these two groups it was possible to build a four-fold table, as follows:

	Cell Size Above 30 c. μ	*Cell Size Below* 31 c. μ
Lambs under one month	12	3
Lambs over three months, and adults	11	88

From this, using Yates modification, $X^2 = 34{\cdot}3$ and P = less than 0·001. Hence the apparent larger average size of corpuscle in young lambs is unlikely to be due to chance.

The remaining attribute shown in Fig. 2 is the erythrocytic fragility, and this can be seen steadily increasing over a period of ten monthly points. This increase in fragility can also be supported by taking the larger group of lambs, where the average maximum fragility is 0·48 per cent. salt, compared to an average of 0·55 for 37 adult sheep. From these groups significance can be tested by a four-fold table with the following cells:—

	Fragility At above 0·5 *per cent.*	*Fragility* 0·5 *per cent. or less*
Lambs under one month ...	3	12
Sheep	26	11

Here, with Yates modification, $X^2 = 6{\cdot}2$, and P is between 0·02 and 0·01. Thus the increased resistance of the erythrocytes in young lambs is shown to be significant.

Leucocytes.—Examining the absolute leucocyte counts given in Fig. 2, it can be seen that the neutrophilic " band forms " are present in greater numbers during the first few months of life. This sign of myeloid activity is not unexpected in actively growing lambs, and it was supported by the examination of other groups of sheep as shown in Table II. Inspection of Fig. 2 also suggested a lower proportion of eosinophil cells in young lambs, and this hypothesis was again supported by the group of 15 lambs which gave an average of 1·1 per cent. eosinophils (S.D. $\pm$ 1·9) in the differential count compared to 4·2 in Fig. 1. This agrees with Fraser, who recorded a lower percentage of eosinophils in lambs. Both the above variations are significant when tested by the " t " test. Further inspection shows an increase in lymphocytes when the lambs were three or four months old, but this change is not supported by the findings in other lambs of that age and individual records for the four lambs on which the figure was based suggests that this result was due to a chance increase in the leucocyte count of only one or two of the individuals from which samples were taken over the four months

TABLE II

BAND FORMS

Age	*No. of Lambs*	*Mean.*
Under 3 months...	20	1·54
3–8 months ...	79	0·94
9 months and over	65	0·51

Percentage of Band Forms in Differential Count for Various Ages.

involved, different individuals showing this change on different occasions. The decrease in the total count during November has also been attributed to chance, for recourse to larger groups showed that 37 lambs of this age group gave an average of 9·5 thousand compared to an average of 9·4 for lambs bled in July or August. Fraser gives figures for lambs at birth and shows that a reversal in the ratios between neutrophils and lymphocytes occurs between birth and the first week, the first samples showing roughly 80 neutrophils to 30 lymphocytes and the second 50 neutrophils to 60 lymphocytes. We have not had an opportunity to confirm this.

Properties of Whole Blood.—The only property of the whole blood recorded in Fig. 2 is that of viscosity, and this can be seen reflecting the variations in the packed cell volume, suggesting that although a correlation could not be shown among samples from different individuals it can be seen where several samples from the same individuals are used.

Seasonal Variation

For the purpose of finding the distribution of the various constituents, histograms were built using coloured cards, marked and cut to denote various attributes. In building histograms of the erythrocytes and their complements, haemoglobin and packed cell volume, it was observed that the lowest values were from sheep sampled in February and March. To avoid this bias, due to this seasonal factor, sheep sampled during the spring, and the month immediately preceding or succeeding it, were not included among the population of Fig. 1 or Table I.

The mean figures for haemoglobin for the four seasons were as follows: Spring, 9·6 g. (26 sheep); summer, 11·7 g. (38 sheep); autumn, 10·6 g. (34 sheep); and winter, 11·7 g. (60 sheep). That the lower figures for February and March were unlikely to be dependent on chance alone can be shown by a chi squared test, with the following cells:—

	Haemoglobin Values above 11·0 *g.*	*Haemoglobin Values below* 11·0 *g.*
Sheep samples in February and March	2	19
Sheep shown in Fig. 1	99	15

Using Yates modification, $X^2 = 52{\cdot}3$ and P is less than 0·001. No other indications of seasonal variation are indicated by Fig. 2, but it will be noticed that the decline in the erythrocytic values in autumn coincides with the lower average of 10·6 for 34 sheep, as compared to 11·7 for the remaining two seasons. This change was not apparent in the first year when the animals were lambs. The group containing sheep bled in February and March consisted of animals kept at Moredun Institute and bled in 1938, 1940 and 1942. The group bled in the autumn consisted of animals bled in 1941 and 1942. This variation is referred to in the discussion.

Variations Due to Sex.

Wirth states that male animals show a higher erythrocyte count and haemoglobin values than females, and that castrated males lie between the two. In building histograms no difference due to sex was noticed and, in addition, we were able to select from our samples 18 pairs of castrated and female sheep, comparable in that they were running in the same flock and were of the same age and breed. Our results, which were obtained in this way from selected sheep, are summarised in Table III, in which the averages for the different constituents in the two groups are given. Comparison shows that the differences between the two groups, if existent, are not of practical importance and that Fig. 1 can be used for either females or castrated males without introducing any great error.

TABLE III

18 PAIRS OF COMPARABLE CASTRATED MALE AND FEMALE ANIMALS

	M.	F.		M.	F.		M.	F.
R.C. ...	11·3	10·5	Eosin.	3·5	4·0	P.C.V. ...	30	30
W.C. ...	12·3	9·7	Bass. ...	0·7	0·53	Visc. ...	5·4	5·8
Band ...	0·75	0·33	Coag....	2·2	2·3	Hb ...	12·1	11·7
Poly. ...	21	22	Frag. ...	0·55	0·6	M.C.V. ...	27	29
Lymph.	71	70	S.G. ...	1052	1052	M.C.H.C.	40	39
Mono.	2·75	2·85						

Variation Due to State of Exertion.

It was thought desirable to find out whether sheep caught quietly in a small pen showed any variation in haematological values from those driven in from near-by fields and excited to some extent by the efforts of a sheep dog. Three sheep were therefore bled with the minimum of excitement, and then chased round a field by an active sheep dog for three minutes. The panting sheep were bled as soon as they returned to the pen, and re-bled an hour later. There was little or no change in any of the constituents shown in Fig. 1 nor was the number of band forms increased.

Variation Due to Breed

In building histograms for the various attributes of sheep's blood no difference due to breed was observed.

DISCUSSION

The only definite disagreement between our results and those of Fraser is the ratio of neutrophils to lymphocytes, for he recorded that neutrophil cells averaged 39 per cent. of the differential count compared to our figure of 24 per cent. The possibility exists that his sheep were suffering from a heavier worm infestation than ours; the complementary explanation that our sheep were suffering from a neutropenia due to some chronic debilitating condition is unlikely in view of the various ages and environments of the sheep used in our investigation. Fraser's method of examining smears is not given, but our own occasional observations confirm those of Hay (1942) that an uneven distribution is unlikely to cause marked error. The possibility that neutropenia is an effect of the colder environment is not substantiated by any definite seasonal variation in Fig. 2. Fraser does not state the breed of his sheep, but it seems unlikely from our experience that this would be responsible for the difference.

Lambs on the day of birth have not been used in this investigation, but Fraser shows a decrease in erythrocytes from 12 million at birth to 10·5 after one week, and quotes other authors who record a higher count for lambs. Owing to the seasonal variation shown

by our sheep in February, March and April, we were unable to add any useful evidence to this contention, except to say that while the adult sheep showed unexpectedly low haemoglobin figures for these months, the figures for lambs born during March and April were much higher (due, as has been explained, to the fewer but larger-sized erythrocytes). Thus, if no seasonal variation existed, presumably the figures for lambs would exceed those found in the adult during summer.

The seasonal variation that has been pointed out has not been investigated and our attempt to explain it is pure speculation. It has been noticed in two instances that pining sheep at Moredun did not commence to thrive until the growth of fresh grass at the end of May or in the beginning of June even although the factor causing their poor condition was removed some months previously. June or July therefore appears to be the time of nutritional abundance when the haemoglobin content of the blood reaches its highest absolute level. With autumn the optimum period terminates and a slight decline in the haemoglobin value commences. In winter the autumnal fall is followed by a slight rise but this is not sustained, a much greater fall in haemoglobin being observed in spring. Climatic conditions during these months make it impossible to keep the feeding at an optimum level and the fall in spring is most probably due to inanition since the rise in haemoglobin commences with the flush of early summer grass. Some proof of this is vouchsafed by the higher levels of haemoglobin shown in Fig. 2 during the spring of 1943, which was mild, and followed an exceptionally mild winter.

SUMMARY

To set standards for healthy sheep so as to facilitate the interpretation of alterations due to disease, samples from 171 sheep were examined. The distributions for animals sampled during the summer, winter and autumn seasons were recorded and are shown in Fig. 1 and Table I.

After a short trial the following estimations were discarded as having little practical value: sedimentation rate; counting of platelets; and the estimation of Price-Jones curves. It was noted that the fragility test used was open to criticism as the variation of 0·05 per cent. NaCl between tubes limited healthy sheep to only six variates. The desirability of placing Schilling's "Degenerative band forms," and other lobular but unsegmented neutrophils, among the adult group when making a nuclear index, is pointed out.

Lambs under one month old were shown to have erythrocytes more resistant to haemolysis with salt solution and of a larger mean corpuscular volume; the probability of lambs having a higher erythrocyte count is discussed. Lambs showed a higher percentage of band form neutrophils and a lower percentage of eosinophils.

A seasonal variation was observed in that during the spring of some years a significant decrease in haemoglobin occurred. No

difference in the blood picture was proved between females and castrated males, resting animals and those just previously exerted, or among the different breeds used in the investigation.

The results showed no disagreement of a practical nature with those of Allcroft for the haemoglobin values of ewes in England and Wales, or with Fraser's blood-picture for English sheep, with the exception of the neutrophil-lymphocyte ratio, which it is speculated, may perhaps have been due to a larger worm burden among Fraser's sheep.

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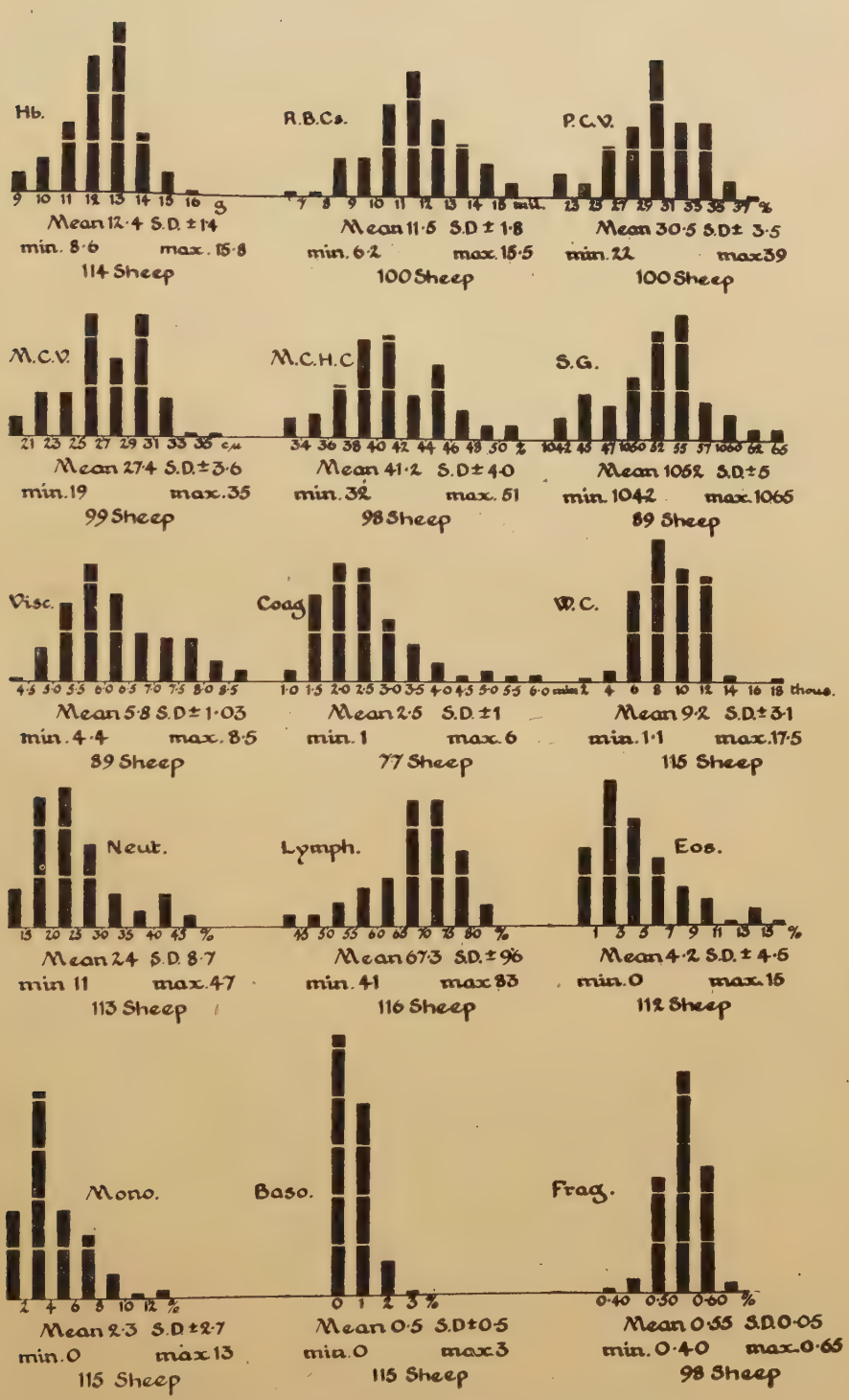

FIG. 1.—Distribution of the principal constituents of the blood of sheep between the months of June and December. White lines mark levels of 10 per cent.

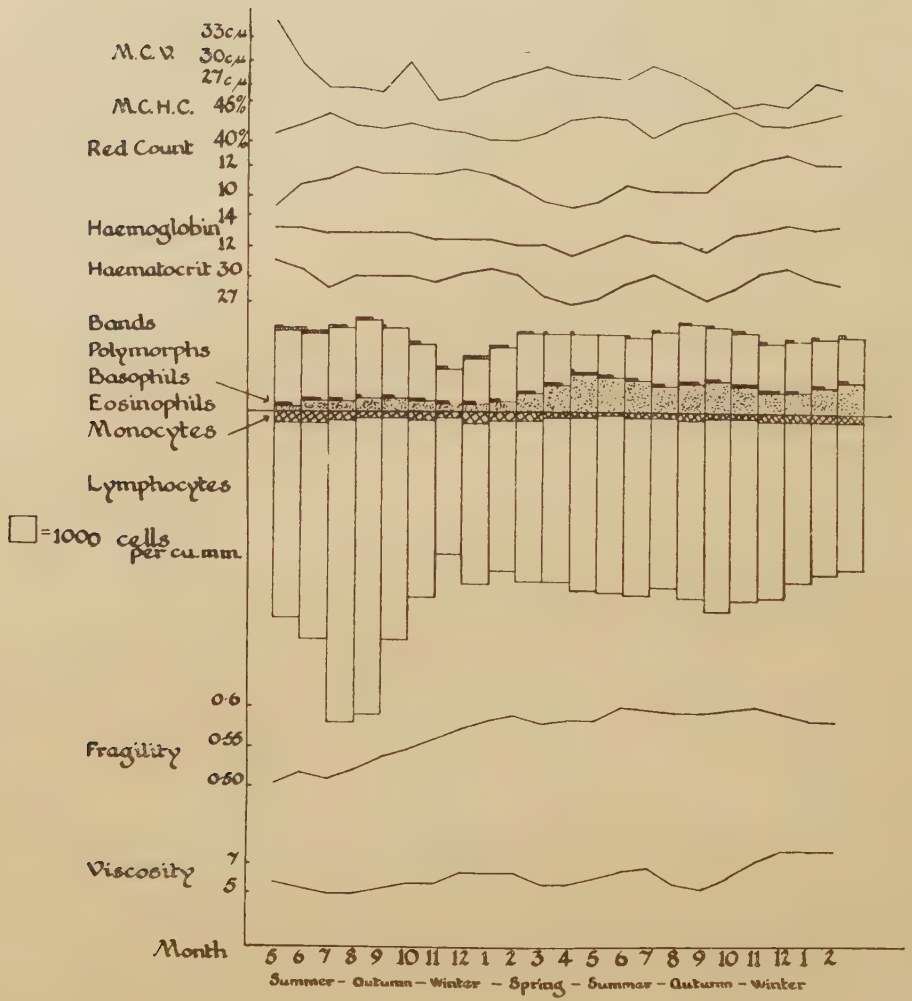

FIG. 2.—Three-month moving averages for four sheep over a period of 24 months. First samples taken when the animals were under one month old.

THE BLOOD PICTURE OF "PINING" CONDITIONS IN SHEEP

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INTRODUCTION

The blood picture of three sheep "pine" diseases, namely, Worm Infestation, Cobalt Deficiency, and Solway Pine, was investigated to ascertain whether differential diagnosis could be obtained. The symptoms in all three diseases are those of progressive debility: growth is retarded, soon after weaning lambs appear stunted and unthrifty, the wool becomes broken, a serous discharge from the eye is common, emaciation and weakness progress until the animal in a state of extreme asthenia is unable to rise, and it then usually dies. Correct diagnosis is a matter of great importance since losses are large despite routine anthelmintic treatment and the feeding of cobalt supplements in those areas where soils are known to be deficient in cobalt.

The methods used in blood examination have been described in previous papers by the authors (Stewart and Holman, 1940; Stewart, Gordon and McCallum, 1940; Holman and Pattison, 1941).

The data obtained in the present investigation are summarised in Table I, wherein the mean values and the standard deviation from the mean are presented as these figures furnish sufficient evidence to indicate the range of variation in concentration of each of the blood constituents.

NORMAL SHEEP

The values are given in the Table for the chemical constituents of the blood of healthy lambs obtained from various parts of Scotland. From the comparative standpoint it may be stated that they were of the same age, *circa* six months, and were fed on a similar diet, namely, summer grass, to the "pining" lambs. The cytological picture and the normal variation in physical constituents of the blood were determined in animals that were more varied both in regard to age and diet, and included most of the above 40 lambs used for determining the chemical picture, as well as some adults.

Most of the values obtained are in keeping with those recorded by other authors. A wide range of concentration of most of the blood constituents and especially of the sugar, for which the mean was 61 mg. per 100 ml. with a S.D. of 10·8, is displayed.

WORM INFESTATION

Worm infestation is found in the greatest number of "pining" sheep. When it is not the primary it is often a secondary cause of much of the debility. As is well known, sheep in a weakened state

TABLE

THE BLOOD-PICTURE

	Normal Sheep			*Worm Infestation*			*Cobalt*		
							Healthy Lambs off same Farms		
	No. of Animals	*Mean Value*	*Stand. Dev. from Mean*	*No. of Animals*	*Mean Value*	*Stand. Dev. from Mean*	*No. of Animals*	*Mean Value*	*Stand. Dev. from Mean*
Sugar (mg. per 100 ml. blood) ...	40	61·0	±10·8	20	62·5	±14·1	10	37·1	±9·1
Hb. (g. per 100 ml. blood)	40	11·2	±1·1	20	6·9	±1·61	10	11·3	±0·48
Cl. (mg. per 100 ml. blood) ...	40	507·0	±20·7	18	543·0	±58·4	10	494·0	±11·1
Ca. (mg. per 100 ml. serum) ...	40	10·7	±0·65	17	9·5	±0·85	10	9·8	±1·1
K. (mg. per 100 ml. serum) ...	40	20·5	±1·84	—	—	—	—	—	—
Na. (mg. per 100 ml. serum) ...	25	34·0	±11·2	—	—	—	—	—	—
Inorg. P. (mg. per 100 ml. blood)	40	5·3	±0·52	18	4·9	±0·99	10	7·0	±0·9
Total P. (mg. per 100 ml. blood)	40	9·9	±0·94	17	9·6	±1·2	10	13·5	±1·6
N.P.N. (mg. per 100 ml. blood)	40	33·3	±5·3	19	39·9	±13·1	10	44·9	±3·9
Amino N. (mg. per 100 ml. blood)	40	7·3	±0·47	18	7·4	±1·64	10	11·5	±1·7
R. B. C.s million per c.mm. ...	100	11·5	±1·8	16	9·2	±2·9	10	13·1	±1·5
Pkd. cell vol. per cent.	100	29·0	±4·0	15	22·0	±4·9	10	31·0	±2·6
M.C.V. (c.μ) ...	100	27	±3·6	15	26	±6·4	10	24	±3·3
M. C. H. C. per cent.	100	41·0	±4·0	14	34·0	±5·8	10	42·0	±3·1
Fragility per cent. NaCl	100	0·55	±0·04	10	0·50	±0·04	10	0·50	±0·04
S.G. aq.=1,000...	100	1,052	±5	—	—	—	10	1,055	±2
Viscosity ...	100	5·8	±1·0	10	4·2	±1·3	10	6·5	±1·2
W.C. thous. per c.mm.	100	9·2	±3·1	17	8·7	±3·4	10	10·6	±1·8
Polymorphs per cent.	100	24·0	±9·0	17	52·0	±16·0	10	34·0	±10·0
Lymphocytes per cent.	100	68·0	±10·0	17	43·0	±15·0	10	55·0	±9·7
Monocytes per cent.	100	3·0	±2·7	17	4·0	±3·1	10	7·0	±1·7
Eosinophils per cent.	100	4·0	±4·5	17	0·7	±1·4	10	3·0	±2·7
Basophils per cent.	100	0·5	±0·5	17	0·2	±0·9	10	1·0	±0·9
Coagulation time (min.)	100	2·5	±1·0	8	2·5	±1·0	10	2·0	±0·4
Liveweight lb. ...	—	—	—	—	—	—	10	78	±7·8

OF PINING CONDITIONS

Deficiency			Solway Pine					
Pining Lambs			Healthy Lambs off same Farms			Pining Lambs		
No. of Animals	Mean Value	Stand Dev. from Mean	No. of Animals	Mean Value	Stand Dev. from Mean	No. of Animals	Mean Value	Stand. Dev. from Mean
8	26·9	±1·9	22	50·5	±8·2	22	42·4	±15·3
8	10·6	±0·6	22	11·7	±0·9	22	9·2	±1·9
8	516·0	±19·9	22	498·0	±16·9	22	510·0	±20·6
10	10·0	±0·7	22	11·3	±0·8	22	10·4	±0·8
—	—	—	22	20·9	±2·6	22	20·2	±3·1
—	—	—	22	34·0	±5·8	22	34·1	±9·9
8	7·2	±1·0	22	5·3	±1·1	22	4·9	±1·3
8	12·8	±1·8	22	11·1	±2·2	22	11·2	±3·1
8	44·2	±5·5	22	35·4	±8·2	22	34·3	±5·3
8	12·0	±2·1	22	9·2	±3·8	22	7·4	±1·9
10	11·6	±1·9	21	12·2	±1·6	22	10·3	±2·4
10	30·0	3·7	22	33·0	±2·7	22	27·0	±5·0
10	26	3·8	21	28	±2·4	22	30	±3·9
10	39·0	3·2	22	38·0	±2·7	22	40·0	±5·5
10	0·50	±0·04	22	0·55	±0·3	22	0·55	±0·02
10	1,053	±5	22	1,050	±4	22	1,046	±5
8	5·2	±0·6	22	5·8	±1·1	22	5·0	±4·6
10	—	—	22	9·5	±4·8	22	7·1	±3·1
10	37·0	±12·0	22	24·0	±6·5	22	38·0	±15·0
10	53·0	±12·0	22	72·0	±6·0	22	58·0	±14·0
10	7·0	±1·6	22	2·0	±1·4	22	2·0	±1·6
10	2·0	±0·8	22	2·5	±2·5	22	1·5	±2·0
10	1·0	±0·6	22	0·5	±0·5	22	0·2	±1·0
10	1·8	±0·4	22	2·0	±0·6	22	2·7	±0·7
10	45	±2·8	—	—	—	—	—	—

are less resistant to worm infestation. Cases of pining, or of very emaciated sheep, are represented in Table I. They were classified after *post-mortem* examination as suffering from heavy worm infestation, either parasitic gastritis or parasitic enteritis, or both combined.

Comparing the results with those of normal lambs, close agreement is to be observed in most of the chemical constituents except in the haemoglobin and chlorides. (The sodium and potassium contents of the blood were not assayed.) A hyperchloraemia would appear to be a usual feature of worm infestation, for many of the cases show an exceptionally high blood chloride value. This probably reflects an upset of the free hydrochloric acid content in the stomach attributable to parasitic action. The mean haemoglobin content of the blood of the worm-infested lambs was only 61 per cent. of that of normal lambs. The range of values was great and in most cases the values were extremely low.

Of the 17 sheep affected with various types of worm infestation seven were definitely anaemic. In three which were suffering from parasitic gastritis the anaemia consisted of an oligocythaemia with regenerative changes in the form of polychromasia and punctate basophilia, indicating an anaemia of the post-haemorrhagic type. In the other four (in three of which *Haemonchus contortus* and in one of which *Chabertia ovinus* predominated) a definite hypochromic normocytic anaemia was apparent. There was little abnormality in the other physical properties except that the viscosity was low.

There was a mild leucocytic reaction for, except in one case, the number of polymorphonuclears exceeded the mean for healthy animals. In six cases there was a neutrophilic shift to the left and in only two were metamyelocytes absent, which is the usual finding in healthy sheep. Eosinophils were well below normal, and were absent in 12 out of 17 counts. This picture of a slight neutrophilia with eosinopenia in parasitic gastritis has already been described by Fourie (1931), Fraser (1930) and Holman and Pattison (1941), but in contrast to the findings of these workers no macrocytic anaemia was observed in any of the cases examined.

COBALT-DEFICIENCY PINE

In many parts of Scotland, more especially Easter Ross, Ayrshire and Roxburghshire and several of the islands off the West Coast, pining in sheep has been definitely traced to cobalt deficiency. In Easter Ross the cobalt status of both soil and pasture on farms where the condition prevails is very low (Stewart, Mitchell and Stewart 1941). Controlled feeding experiments have shown that the pining on such pasture is due to cobalt deficiency *per se* (Stewart *et al.*, 1941). In many areas the application of cobalt-rich manures has completely prevented the occurrence of pining in flocks on farms where it has prevailed for many years. On some farms in these areas the worm burden of sheep is remarkably low. In these areas parasites would appear to play only a secondary role. It has

been recently emphasised (Lyle Stewart and Crofton, 1943) that trace element deficiency and worm infestation are equally responsible for " pine " in sheep in Northumberland, but, unfortunately, the cobalt content of the soil and pasture is not given and so it is impossible to compare the degree of cobalt deficiency in the soils of Northumberland with that in those of Ross-shire.

During an experiment the results of which have already been reported (Stewart *et al.*, 1941), one group of 30 lambs was grazed on a cobalt-rich pasture while another group of 30 was on cobalt-deficient pasture. At the age of four months, by which time the two groups were showing a remarkable difference in body weight and physical condition, samples of blood and faeces were obtained from ten lambs in each group. (Unfortunately on arrival at the laboratory only eight blood samples from the cobalt-deficient group were in a suitable condition for chemical analyses.) The mean weight of the cobalt-fed lambs was 78 lb. and of the cobalt-deficient lambs, 45 lb. This is a significant difference inasmuch as both groups were of equal age and had been chosen at random from the flock on the farm at the beginning of the experiment.

From the distribution of the faecal worm egg counts (Table II) it is concluded that the worm burden of both groups was about equal and not heavy enough to cause much debility.

TABLE II

DISTRIBUTION TABLE OF FAECAL WORM-EGG COUNTS OF LAMBS SUFFERING FROM COBALT DEFICIENCY AND SOLWAY PINE

		0–499 *per g. faeces*	500–999 *per g. faeces*	1,000–1,999 *per g. faeces*	2,000–3,999 *per g. faeces*	4,000–5,999 *per g. faeces*	6,000 and *over per g. faeces*
Cobalt-Deficiency Area	Affected lambs	1	4	1	1	–	1
	Healthy lambs off same farm	3	2	3	1	–	–
Solway-Pine Area	Affected lambs	2	1	3	7	4	3
	Healthy lambs off same farms	4	1	8	3	3	–

If the blood picture of the cobalt-fed, healthy lambs is compared with the blood picture of normal lambs from other areas, the most interesting differences are the rather low blood sugar and the higher blood phosphates of the former (Ross-shire) lambs. It will be observed that for haemoglobin the mean value is almost identical and the standard deviation from the mean is smaller in the case of the Ross-shire lambs. The non-protein-nitrogen and amino nitrogen contents of the blood are slightly higher. In some degree

the variations observed may be due to the type of pasture on which the lambs were grazing.

The blood picture of the pining lambs on the cobalt-deficient pasture shows variations from the blood picture of normal lambs for the most part similar to those observed in the case of the cobalt-fed healthy lambs; the blood sugar was even lower with a much smaller deviation from the mean. It is only for the haemoglobin that any difference can be seen between healthy and pining lambs on the same farm. The mean values were 11·3 g. and 10·6 g. respectively, but it will be observed that 10·6 g. is still within the range of normality. It is not nearly so low as the haemoglobin of lambs suffering from worm infestation. The red cell count of the pining lambs while slightly lower than that of the cobalt-fed lambs, was higher than that regarded as normal for the species. It must be understood that although these cobalt-deficient lambs showed marked retardation in growth and listlessness, they were not in the manifest clinical condition covered by the term "pine."

Therefore it would appear that cobalt deficiency in itself does not cause anaemia nor does it materially interfere with haemoglobin formation. Anaemia only becomes apparent as the lambs develop severe debility. The view would, therefore, be disproved that the function of cobalt in metabolism is linked with haemoglobin formation, a belief which seems to have arisen when cobalt deficiency was thought to be related to iron deficiency. The results substantiate the findings of Robscheit-Robbins and Whipple (1942), who found that cobalt did not affect haemoglobin formation. It would appear that cobalt plays some other physiological role, one probably more intimately associated with growth since mature sheep do not seem to suffer from cobalt deficiency.

SOLWAY PINE

This is a "pining" condition, the symptoms of which closely resemble those of parasitic infestation and cobalt deficiency. As far as can be ascertained the condition is localised to the foothills of the Criffel Mountain from New Abbey to Kirkgunzeon and to many farms in the Auchencairn district near the Solway Firth. Mortality is high and the disease has led to cessation of sheep breeding in several of the districts where it was most prevalent. Instead, farmers have resorted to "flying stocks" since adult animals are not susceptible to the disease. It affects lambs at a much younger age than does cobalt deficiency or worm infestation. Severe clinical symptoms are obvious soon after weaning and by the end of July large numbers of lambs are seriously affected. Farmers aver that the symptoms are obvious long before weaning.

The disease appears to be related to granitic soils which are very common in the districts mentioned above. Soil and pasture analyses reveal that both are very low in minerals, especially magnesium

and potassium. In at least one of the areas, the cobalt content of the pasture is low but what rôle, if any, cobalt deficiency plays in the etiology of the disease has yet to be determined. Experimentation along the line of mineral feeding is in progress.

Towards the end of July samples of blood and faeces for examination were taken from 22 affected lambs and from 22 of the healthiest lambs on three farms in the Kirkgunzeon area. The results are shown in Tables I and II. Worm egg counts show that the lambs on these farms were carrying a rather high worm burden, but that in only a few of the affected lambs was it higher than in the apparently normal lambs. It was observed that those lambs showing the severest clinical symptoms of pine or debility were those which had the highest faecal worm egg counts but, as has already been mentioned, this is a frequent finding in debilitated animals. Since the egg count distribution curve of the healthy lambs is similar to that of the pining lambs except in the case of those showing severest symptoms, it is inferred that the primary cause of the pining is not worm infestation. This view is to a certain extent confirmed by the fact that pining is just as prevalent on those farms in these districts where routine anthelmintic treatment is carried out as on those where no anthelmintics are given.

From Table I it will be seen that the mean values of all the chemical constituents of the blood of the healthy sheep on the Solway farms are in close agreement with those of normal sheep. The greatest difference is seen in the values of blood sugar, namely, 50·5 mg. compared with 61 mg. It will be noticed that even the mean value of 50·5 mg. is well above the mean of the blood sugars of the healthy Ross-shire lambs (37·1 mg.). In the case of the pining Solway lambs the blood sugar was much lower than the normal, being of the order of the Ross-shire lambs, and showing a large variation amounting to a S.D. of 15·3, around a mean value of 42·4 mg.

The greatest divergence from the normal was observed in the haemoglobin values of the pining lambs. A mean value of 9·2 g. with a S.D. of 1·9 was found for the latter as compared with a mean of 11·7 and a S.D. of 0·9 for the healthy lambs on the same farm. The large standard deviation shows that the range was very wide, the haemoglobin values of a few of the lambs being less than 7 g. It is probable that the fall in haemoglobin in these few lambs was associated with the worm infestation or general debility. To some extent this is confirmed by the individual records of the physical properties, since in most cases in the affected group the low haemoglobin figures were related to a simple oligocythaemia except in two lambs which showed a definitely hypochromic normocytic anaemia (M.C.H.C. 29·8 per cent. and 30·8 per cent.). The worm egg counts of these two sheep were 12,780 and 5,200 per g. respectively.

Changes in the other constituents of the blood were insignificant and the slight changes in the physical properties were probably due to the decrease in red cell volume.

CONCLUSIONS

The results do not give promise that blood analyses will be of much service in differentiating the three types of "pining" discussed. In sheep affected with a heavy worm infestation or severe debility there is an anaemia with an appreciable neutrophilic reaction. A simple hypochromic anaemia arising from other causes is, however, known to occur in sheep. On the other hand, where there is no fall in the blood haemoglobin and red cell count in cases of severe "pining," the cause is unlikely to be worm infestation and it may be cobalt deficiency.

As more knowledge upon the cobalt status of soil and pasture is forthcoming, and the soil properties and degree of pining can be correlated, a soil and pasture analysis will be a better guide to the type of pining present than will be blood examination.

The data presented in this paper do not lend support to the theory that the physiological role of cobalt is associated with haemoglobin formation.

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A STUDY OF THE THERAPEUTIC ACTION OF THE PHENANTHRIDINIUM COMPOUND 897 AGAINST *TRYPANOSOMA CONGOLENSE* INFECTION IN CATTLE

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INTRODUCTION

The substance 7-amino-9 (*p*-aminophenyl)-10-methyl-phenanthridinium chloride was discovered by Morgan and Walls (1938). Browning *et al.* (1938 and 1940) have investigated its trypanocidal action against various strains of *T. congolense* in mice and reported favourably on its action.

The object of the experiments reported in this paper was to ascertain its value against *T. congolense* in cattle, with a view to assessing it as a therapeutic agent under field conditions which demand that (*a*) it must be easily administered, *e.g.*, by African assistants; (*b*) it must be of low toxicity since dosage can only be computed on estimated weight; (*c*) the dose of the drug should be preferably in a small volume; (*d*) a single dose should, if possible, bring about "cure."

The two strains of *T. congolense* used for the experiments (Kikaya and Buruli), were so named from the localities in Uganda where they were first isolated. A 1 per cent. solution of the drug was used throughout since more concentrated solutions could not be obtained owing to its low solubility.

TOXICITY

To determine the toxicity of the drug for cattle, healthy animals were given varying doses intravenously (Table I).

Table I

No.	*Dose. Mg./kilo. Body Weight*	*Remarks*
5118	4·5	Muscular contractions during injection, tongue protruded, penis partially erected, micturated. Required 10 minutes to rise, walked with unco-ordinated movements, laboured breathing.
5212	4·5	No apparent effects. Got up immediately.
5183	3·5	Showed muscular spasm immediately after injection, increased heart rate. Required 5 minutes to rise, tongue protruded, laboured breathing.
5193	3·5	No apparent effect.
5168	3·0	„ „
5172	3·0	„ „
5133	2·5	„ „
5159	2·5	„ „

The highest non-toxic dose intravenously thus proved to be 3 0 mg./kilo body weight. Owing to the low solubility of the drug, the volume of this dose was considerable and, for general use, hardly practicable.

A physical examination of the blood of the above cattle was carried out at two-weekly intervals before and at three-weekly intervals after administration of the drug. No appreciable changes were found in the red and white cell counts, haemoglobin, colour index, or cell volumes. Chemical examinations of the blood at the same intervals failed to show significant changes in the glucose, chloride, nitrogen, carbon dioxide and phosphorus values.

TREATMENT

In a preliminary experiment, cattle were infected with *T. congolense* and then treated with 2 mg./kilo body weight of the drug. All were in the chronic stages of the disease (Table II). No attempt was made to determine the effect of treatment during crises because most cattle requiring treatment in the field are in the chronic stages of the disease. Cure was considered to have occurred when the animals were well, the blood remained free from trypanosomes on daily examination for a period of four months and subinoculations into susceptible cattle proved negative.

TABLE II

No. of Animal	*Result*
5123, 5137, 5151, 5176	"Cured." Peripheral blood clear to microscopic examination after 4 days and subinoculation later into susceptible animals gave negative results.
5207, 5136	Infected controls.
5145, 5208	Normal controls.

The infected animals all showed a significant fall in haemoglobin and red blood corpuscle values, otherwise there was no appreciable change. In the treated animals the haemoglobin returned to normal within two weeks whilst the red cell value recovered completely after 12 weeks. The controls were included mainly as a check against the blood examinations which were carried out at weekly intervals.

To discover the most effective route of administration and amplify the above results, a herd of infected cattle was divided into groups of eight animals and treated with a dose of 2 mg./kilo body weight. Each group contained four animals infected with the Buruli strain and four with the Kikaya strain (Table III).

TABLE III

Route of Administration	*Result*
1. Intravenous	3 cured, 4 relapsed, 1 death intercurrent infection.
2. Subcutaneous	2 cured, 5 relapsed, 1 death intercurrent.
3. Intramuscular	1 cured, 7 relapsed.
4.* Intravenous and subcutaneous	1 cured, 7 relapsed.
5.* Intravenous and intramuscular	4 cured, 4 relapsed.

* Half the total dose was given by each route.

The initial treatment cured eleven animals, 27 relapsed and two died from intercurrent conditions. All the relapses occurred from 14 to 29 days after treatment. The 27 cases of relapses were all re-treated by a single intravenous injection at the rate of 2 mg./kilo administered 44 days after the first treatment; 22 of them were then cured. Five animals all infected with the Buruli strain, which relapsed after the second treatment, were similarly re-treated again after a further period of 49 days, and they again relapsed. A fourth treatment after a further 28 days period again proved ineffectual. The trypanosomes in these refractory cases appear to have become drug-fast.

The intravenous route, or the intravenous and intramuscular routes combined, proved to be the best. The other routes or combinations of routes proved ineffectual with a dose of 2 mg./kilo. Administration of the drug intramuscularly caused stiffness and lameness with a slight degree of local inflammatory reaction.

PROPHYLAXIS

To ascertain the prophylactic value of the drug animals were given the drug intravenously in a dose of 2 mg./kilo body weight and subinoculated with the Kikaya strain at varying intervals. (Table IV.)

TABLE IV

Subinoculated with T. congolense		*Result*
Days after Administration of Drug	*Number of Animals*	
1	2	Not infected.
2	2	1 not infected.*
4	2	Both positive after 10 days.
6	2	„ „ 14 „

Control animals which had not been treated were inoculated at the same time as the subinoculations were made and they all showed *T. congolense* in the peripheral blood after about 7 days.

* The second animal showed *T. congolense* in its peripheral blood after 30 days and was thought to have become naturally infected by mechanical transmission.

These results show that the drug protects against a very heavy infection of *T. congolense* up to 48 hours after administration.

DISCUSSION

From these few experiments, the phenanthridinium compound 897 was proved to have a useful therapeutic action upon *T. congolense* infection in cattle. From experience it must thus be given a high place among the trypanocides tried against this very intractable infection. The drug is of low toxicity, but its low solubility precludes saturation dosage as a routine measure. Definite drawbacks are the volume required for adequate dosage, and the high percentage of relapses which follow the first injections. It is, however, worthy of more extended use. If it could be rendered more highly soluble its therapeutic value would be enhanced. As a prophylactic, it may prove useful for protecting trade cattle from infection during short journeys of not more than 24 hours through fly belts.

SUMMARY

The toxicity of the phenanthridinium compound 897 has been investigated. The therapeutic and prophylactic value of the drug in *T. congolense* infection in African cattle is recorded.

ACKNOWLEDGMENT

The phenanthridinium compound used in these experiments was supplied to us by the Chemical Research Laboratory of the Department of Scientific and Industrial Research, Teddington, Middlesex, where it was prepared.

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THE BEHAVIOUR OF SOME SMOOTH AND ROUGH STRAINS OF *BRUCELLA ABORTUS* IN THE DEVELOPING CHICK EMBRYO

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INTRODUCTION

The fact that *Brucella abortus* is capable of invading the chick embryo was observed by Goodpasture and Anderson (1937). Further information regarding the behaviour of the parasite was provided by Buddingh and Womack (1941), who found that heavy inocula (a loopful of culture) of *Br. abortus* would cause death of 12-day-old chick embryos in from three to four days with involvement of the liver, spleen, myocardium and kidney. These workers described the route of invasion followed by the parasite, its intracellular habit of growth and its preference for endothelial cells.

The effect of the virulence of a strain of *Br. abortus* on its behaviour in the chick embryo was studied by Metzger and Stokes (1940), who compared the infection produced by a fully virulent field strain of the organism with that produced by a smooth strain of reduced virulence (strain 19). They found that both were equally capable of invading the embryo and causing its death. Invasion was shown to result from the inoculation of as few as six organisms. They concluded that " the chick embryo acts as a passive sort of medium which offers little or no resistance to the growth of *Br. abortus*." In both cattle and guinea-pigs, however, strain 19 behaves as a strain of very low virulence and the difference between its behaviour and that of a strain of high virulence is very great.

The experiments to be described were undertaken to throw further light on the behaviour of avirulent strains of *Br. abortus* in the chick embryo.

Experimental Methods

(1) *Strains of Br. abortus Employed.*—Five strains of *Br. abortus* were studied in the course of these experiments.

Strain " 544 " (McEwen) is a smooth strain of high virulence both for cattle and guinea-pigs, the characteristics of which have been described by McEwen (1940).

Strain 19.—A smooth strain of very reduced virulence for guinea-pigs. (Cotton, Buck and Smith, 1933.)

Strain 45 (20) (McEwen).—A rough strain of reduced virulence for guinea-pigs (McEwen, 1940)'

Strain " *Sparrow*."—A rough strain of reduced virulence.

Strain " *Jones*."—A rough strain of reduced virulence.

(2) *Size of Doses.*—Doses of three different magnitudes were used for inoculating the chick embryos. They were prepared by

washing off with Ringer's solution the growth from a 48-hour culture of the required strain of *Br. abortus* grown on bacto-tryptose agar, and by standardising the opacity of the resulting suspension to match number two tube, Brown's scale. This suspension was diluted ten thousandfold and a millionfold. The amounts of these suspensions inoculated into each of the groups of developing eggs and the approximate numbers of viable organisms they contained were as follows :—

Group I received 0·2 c.c. of Brown tube 2 suspension containing 600 million viable organisms.

Group II received 0·1 c.c. of $\frac{\text{Brown tube 2}}{10{,}000}$ suspension containing 30,000 viable organisms.

Group III received 0·1 c.c. of $\frac{\text{Brown tube 2}}{1{,}000{,}000}$ suspension containing 300 viable organisms.

(3) *Method of Inoculation.*—The embryos were inoculated after nine days' incubation. An artificial air space was created by the method described by Burnet (1936). The required amount of infective suspension was dropped on to the surface of the exposed chorio-allantoic membrane by means of a pipette delivering 0·02 c.c. per drop.

(4) *Determination of Time to Death of Embryo and Recovery from it of Infective Organism.*—The inoculated embryos were incubated at 37° C. and observed daily through the glass covering which sealed the opening in the shell. Cessation of movement of the embryo was taken to indicate death. Each dead embryo was removed from its shell and its liver and spleen spread on bacto-tryptose agar slopes. The amount of growth of *Br. abortus* obtained after incubation of such slopes was indicated by plus signs : ++++ = confluent growth, +++ = almost confluent, ++ = over 100 colonies ; + = less than 100 colonies.

(5) *Distribution of* Br. abortus *Within the Host Tissues.*—A microscopic study of the distribution of *Br. abortus* within the host tissues was also made. Portions of the chorio-allantoic membrane were attached to slides by means of egg albumin and stained, when dry, with Ziehl Nielsen carbol fuchsin (stretch preparations). The material was then partly decolourised with 0·5 per cent. citric acid and counterstained with methylene blue. By this means differential staining of the organisms within the cells was obtained, the cells being stained blue, the organisms red. A similar treatment was applied to smears prepared from the livers, spleens, kidneys and brains of infected embryos. The allantoic fluid and yolk material were also investigated by this means.

Since it was observed that considerable splenic enlargement resulted from the infection of the embryo with *Br. abortus*, a special

series of embryos was inoculated to provide material for the histological study of such enlarged spleens. The spleens were removed after the deaths of the embryos, fixed with Bouin's solution and stained by the method described above.

Experimental Results

Table I records the times which elapsed between inoculation and death of embryos inoculated with different doses of the five strains of *Br. abortus* studied in these experiments. Only those embryos from the tissues of which the organism was recovered were included in the table. Strain 19 was tested on two separate occasions,

Table I

Time to Death of Chick Embryos Inoculated when Nine Days Old with Various Doses of Different Strains of *Brucella abortus*

Strain	Dose Viable Organisms	Number of Embryos Inoculated	Days after Inoculation												Per cent. Deaths	Per cent. Hatching
			1	2	3	4	5	6	7	8	9	10	11	12		
544 ...	600 million	10	0	0	0	4*	6	–	–	–	–	–	–	–	100	0
19 (Test I)	„	9	0	1	2	5	1	–	–	–	–	–	–	–	100	0
19 (Test II)	„	10	0	0	1	9	–	–	–	–	–	–	–	–	100	0
45 (20) ...	„	10	0	1	0	1	1	0	0	0	0	0	0	2	50	30
Sparrow...	„	10	0	0	0	0	0	0	0	0	0	0	0	–	0	60
Jones ...	„	10	0	0	0	0	0	0	0	0	0	0	2	–	20	50
544 ...	30 thousand	6	0	0	0	0	1	0	3	2	–	–	–	–	100	0
19 (Test I)	„	6	0	1	0	0	3	0	0	2	–	–	–	–	100	0
19 (Test II)	„	10	0	0	0	0	1	2	5	–	–	–	–	–	80	20
45 (20) ...	„	5	0	0	0	0	0	0	0	0	0	0	0	0	0	100
Sparrow...	„	9	0	0	0	0	0	0	0	0	0	0	0	0	0	90
Jones ...	„	10	0	0	0	0	0	1	0	0	0	0	0	0	10	100
544 ...	3 hundred	10	0	0	0	0	1	1	0	3	1	1	2	1	100	0
19 (Test I)	„	10	0	0	0	0	0	1	2	3	0	1	0	0	70	10
19 (Test II)	„	10	0	0	0	0	1	2	3	0	0	0	0	0	60	40
45 (20) ...	„	10	0	0	0	0	0	0	0	0	0	0	0	0	0	80
Sparrow...	„	9	0	0	0	0	0	0	0	0	0	0	0	0	0	100
Jones ...	„	10	0	0	0	0	0	0	0	0	0	0	0	0	0	100

* Figures = number of deaths due to *Br. abortus* infection.

the strain being obtained from different sources. The results of these two tests are recorded separately. In Table II the quantities of *Br. abortus* present in livers and spleens of embryos which died as a result of infection are recorded, the quantities of the organism being assessed by plus signs as explained in the previous section.

Table III records the rate of invasion of the chick embryo by *Br. abortus* strain 544, 19 and 45 (20). The embryos in this experiment were inoculated with approximately 30,000 viable organisms of the strain to be tested. They were killed and their spleens and livers cultured at two and four days after inoculation. The amount of infection found in these organs is indicated by plus signs.

TABLE II

AMOUNT OF INFECTION IN SPLEENS AND LIVERS OF CHICK EMBRYOS KILLED BY INOCULATION OF VARIOUS STRAINS OF *Brucella abortus*

Strain	*Size of Dose*								
	600 *Million v. Organisms*			30 *Thousand v. Organisms*			300 *v. Organisms*		
	No. of Embryos	*Liver*	*Spleen*	*No. of Embryos*	*Liver*	*Spleen*	*No. of Embryos*	*Liver*	*Spleen*
544	10	9 ++++ 1 ++	9 ++++ 1 +	6	6 ++++	6 ++++	10	10 ++++	10 ++++
19 (I)	9	9 ++++	9 ++++	6	6 ++++	6 ++++	7	7 ++++	7 ++++
19 (II)	10	10 ++++	10 ++++	8	8 ++++	8 ++++	6	6 ++++	6 ++++
45 (20)	5	1 ++++ 1 +++ 3 ++	1 ++++ 1 +++ 1 ++ 2 +						
Jones	2	2 ++	1 ++ 1 +						

No striking lesions were found in the chorio-allantoic membranes of embryos which had died of *Br. abortus* infection, but there was usually a definite thickening of the membrane which also appeared abnormally opaque. Direct examination of stretch preparations revealed dense masses of bacteria filling cells in the walls of the blood vessels, thus confirming the findings of Buddingh and Womack (1941). A thick fibrinous exudate was usually found between the chorion and allantois. The external appearance of the dead embryo was usually normal; the most marked lesion of the internal organs

TABLE III

THE AMOUNT OF INFECTION IN CHICK EMBRYOS AT TWO AND FOUR DAYS AFTER INOCULATION WITH 30 THOUSAND VIABLE ORGANISMS OF DIFFERENT STRAINS OF *Brucella abortus*

Strain	*Days from Inoculation*					
	Two Days			*Four Days*		
	No. of Embryos	*Liver*	*Spleen*	*No. of Embryos*	*Liver*	*Spleen*
544 ...	10	7 + 3 N	10 N	10	8 +++ 1 + 1 N	5 +++ 3 ++ 1 + 1 N
19 ...	7	1 +++ 2 ++ 3 + 1 N	2 +++ 1 ++ 2 + 2 N	7	2 ++++ 4 +++ 1 +	2 ++++ 2 +++ 2 ++ 1 N
45 (20)	7	1 ++ 6 +	6 + 1 N	7	1 ++ 5 + 1 N	1 ++ 5 + 1 N

++++ = confluent growth. +++ = almost confluent. ++ = over 100 colonies. + = less than 100 colonies. N = Brucella absent.

was a gross enlargement of the spleen, particularly pronounced in those embryos which had received a small infective dose and in which the time to death had been longest. The extent of this splenic enlargement can be judged from Fig. 1, in which the outlines of a median section of an enlarged and a normal spleen are shown. The liver was also usually abnormal in appearance, mottled or partly covered with whitish areas. Microscopical examination of smears of these organs and of the kidney revealed cells swollen out with bacteria to about three times their normal size (Fig. 2), similar to those figured by Buddingh and Womack. In fixed preparations of these organs a similar crowding of certain cells with *Br. abortus* could be observed. In no case could any organisms be discovered within the tissues of the brain, though Metzger and Stokes (*ibid.*) report finding the organism in this site. The allantoic fluid was

always heavily infected with *Br. abortus* but the organism was not found in the yolk.

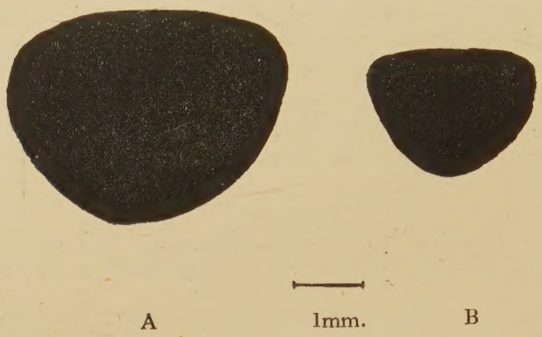

FIG. 1.—Comparative areas of median sections of spleens. A from a chick embryo dead of *Brucella abortus* infection; B from a normal embryo of same age.

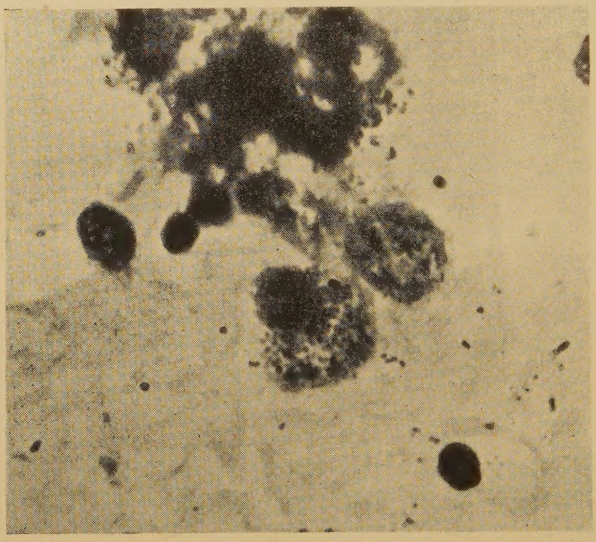

FIGURE II.—Smear of liver of 15 day old chick embryo infected with Brucella abortus (strain 19).

In the centre of the field can be seen a cell packed with organisms. (x 1800).

On a few occasions *Br. abortus* was shown to be present in very small numbers in the livers and spleens of those chicks which hatched out, but there seems no reason for thinking that infection of the embryo will give rise to a persistent infection of the chick if the embryo survives until time of hatching. As soon as the chick hatches, the infection appears to retrogress.

DISCUSSION OF RESULTS

The chief aim of these experiments was to discover whether strains of *Br. abortus* of very different degrees of virulence for the guinea-pig were all of equal virulence for the nine-day-old chick embryo. It is obvious from Table I that the present work confirms the findings of Metzger and Stokes (*ibid.*) regarding the identical behaviour in the chick embryo of the avirulent smooth strain 19 and a fully virulent strain. Although there are some indications that strain 19 kills the embryo a little less readily than does strain 544, the difference is small and cannot be accepted as being significant. It will be observed that the mean time to death is practically identical for both strains and closely related to the size of the infective dose.

On the other hand, the behaviour of the three rough strains investigated presents a clear-cut contrast with that of the two smooth strains. These rough strains are, for the most part, incapable of causing the death of the embryos, even when inoculated into them in overwhelmingly large numbers (600 million organisms).

It may be assumed that the rough strains have little or no capacity for invading the embryo, since, as shown in Table II, strain 45 (20), though capable of gaining access to liver and spleen of the embryo, has been unable to multiply to any extent within these organs, in marked contrast with the smooth strains, both of which have multiplied rapidly.

It is worthy of note, however, that, in the spleen of the guinea-pig, the avirulent rough strain 45 (20) is capable of surviving in small numbers for as long as 12 weeks whereas the avirulent smooth strain 19 is not capable of surviving for as long.

SUMMARY

1. The behaviour in the nine-day-old chick embryo of one smooth and three rough strains of *Br. abortus*, all of reduced virulence for the guinea-pig, was compared with that of a smooth strain of high virulence for the guinea-pig.

2. It was found that the smooth avirulent strain (strain 19) behaved in the chick embryo as a fully virulent strain, causing death of a high proportion of the embryos with enlargement of the spleen and lesions in the liver.

3. The three rough strains all proved incapable of producing a fatal infection in the chick embryo except when inoculated in very large numbers. The data suggested that rough strains of *Br. abortus* are not capable of invading the chick embryo.

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